Comparison of digestion and particle-associated bacteria after *in situ* incubation of different barley varieties in the rumen of cattle

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ABSTRACT

The chemical composition of barley grain, including the structure of starch, can vary among barley varieties and result in different digestion efficiencies. It is not known if compositional differences in barley can affect the particle-associated bacteria (PAB) involved in digestion. Therefore, the objective of this study was to characterize the *in situ* rumen digestion and PAB of four barley grain varieties. Three ruminally-cannulated heifers were fed a low grain (60% barley silage, 37 % barley grain and 3% supplement) or high grain (37% barley silage, 60% barley grain and 3% supplement) diet. Four different barley varieties (Fibar, Xena, McGwire and Hilose) and corn as a control were included in the experiment. A series of rumen incubations were carried out. One set of bags (3 heifers x 3 bags/time point/treatment; n=9) containing 3 g of ground grain was used to estimate dry matter (DM), starch and crude protein (CP) disappearance. A second set of bags (2 heifers x 3 bags/time point/ treatment; n=6) containing 5 g of ground grain were incubated and used for DNA extraction. A third set of bags (2 heifers x 2 bags/time point/treatment; n=4) containing ground grain (5 g) were incubated and examined using scanning electron microscopy (SEM). The same two heifers were used for DNA and SEM bags. Bags to estimate nutrient digestion were incubated for 0, 2, 4, 12, 24 and 48 h and for 2, 4, and 12 h for DNA extraction and SEM. DNA was extracted to characterize PAB via 16S rRNA gene sequencing followed by analysis using QIIME. In the low grain diet, McGwire had the highest effective degradability (ED) of DM ($P<0.01$), followed by Xena, Fibar, Hilose, and corn, respectively. The ED of starch was highest ($P<0.01$) for Fibar, McGwire, and Xena, followed by Hilose and Corn while the ED of protein showed that Corn had lower ED than barley grains. For the high grain diet, Fibar and McGwire had the highest ED of DM ($P<0.01$), followed by Fibar, Hilose and corn, respectively. The ED of starch was highest ($P<0.01$) for Xena and Fibar,

followed by McGwire, Hilose and corn. The ED of protein was highest (P<0.01) for Fibar (55.0%) and lowest for Corn (32.0%). Barley variety did not affect the relative abundance of phyla, but they did differ with incubation time in both the low and high grain diets. However, after 12 h of incubation the diversity of bacteria differed from that after 2 and 4 h of incubation in the rumen with both diets. *Lactobacillus* (approximately 80%) dominanted after 12 h of incubation when cattle were fed low grain diet, with both *Prevotella* and *Lactobacillus* being the most abundant genera after 12 h of incubation with the high grain diet. This study found that the diversity of PAB on barley grain was not affected by barley variety, despite there being differences in digestion kinetics. However, time affected PAB, illustrating that the bacterial biofilm involved in the digestion of grains clearly undergoes compositional shifts during ruminal digestion. Moreover, the digestibility and bacterial biofilm were affected by differences in endosperm structure between corn and barley. This is probably because corn and barley differ in their endosperm structure, especially with regard to the protein matrix, which could affect digestibility and the formation of grain-associated bacterial biofilm.

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LIST OF ABBREVIATIONS

PAB	Particle-associated bacteria
DM	Dry matter
CP	Crude protein
ED	Effective degradability
SEM	Scanning electron microscopy
NGS	Next-generation sequencing
VFA	Volatile fatty acids
NRC	National Research Council
AAFC	Agriculture and Agri-Food Canada
FCDC	Field Crop Development Centre
CDC	Crop Development Centre
NDF	Neutral Detergent Fiber
ADF	Acid detergent fiber
ADG	Average daily gain
TN	Total nitrogen
SDF	Soluble dietary fibre
IDF	Insoluble dietary fibre
OTU	Operational taxonomic unit

1.0 GENERAL INTRODUCTION

Barley is the most common grain used as an energy source in the diets of feedlot cattle in western Canada. It has higher levels of protein than corn resulting in more methionine, cysteine, lysine, and tryptophan in the diet (Nikkhah, 2012). It also is rich in potassium, vitamin A, calcium, copper, molybdenum and manganese, but has lower levels of zinc, vitamin C and vitamin B12 compared to corn (Nikkhah, 2012). These attributes make barley an economically and nutritionally important feed for cattle.

Ruminal digestion of barley grain ranges between 80 to 90 % (Nocek and Tamminga, 1991). The efficiency of digestibility of this cereal grain can be affected by variety (Ramsey et al., 2001) and processing method (Nikkhah et al., 2004; Huntington, 1997). The whole barley kernel is covered by a fibrous hull, which if not breached, reduces the kernel's digestibility in the rumen. This reflects the fact that the hull of the barley kernel resists digestion by rumen bacteria and prevents bacteria from gaining access to the digestible starch granules within the endosperm (Beauchemin et al., 1993; McAllister et al., 1994). Therefore, some barley types (CDC McGwire, CDC Fibar, CDC Hilose) have been developed to be hulless as a means of increasing the efficiency of digestion of barley in both monogastrics (Pettersson and Lindberg, 1997) and ruminants (Zinn et al., 1996). Hulless varieties of barley grain contain less fiber and more protein than hulled varieties, making them more digestible in the rumen (Zinn et al., 1996).

Microbial attachment is the first step in the digestion of starch granules in the rumen. The attached bacteria readily penetrate the damaged areas and colonize the surface of feed particles and release soluble material. Bacterial attachment is an important step in feed digestion in the rumen (Minato and Suto, 1979) and to some extent it is substrate specific. *Streptococcus bovis, Ruminobacter amylophilus, Prevotella ruminicola, Butyrivibrio fibrisolvens,*

Succinimonas amylolytica and *Selenomonas ruminantium* are the known starch-digesting bacteria (Cotta, 1988) and their numbers increase when grain is fed. Therefore, factors such as the chemical composition and physical structure of grain may alter the nature of the rumen bacterial populations that participate in the digestion process.

Our knowledge about rumen microbial ecology is mostly based on culture techniques (i.e., isolation, enumeration and metabolic characterization), an approach that has characterized less than 10 % of the bacteria, which reside in the rumen (McAllister et al., 2006). Therefore, knowledge on the rumen microbial ecology and nature of the biofilms involved in the digestion of cereal grains is limited. However, recent advances in molecular techniques such as the use of DNA microarrays and next-generation sequencing (NGS) technologies are providing new information on the non-culturable bacterial species that contribute to microbial diversity within the rumen. For example, one study showed that a shift in bacterial populations occurs over the time course of fiber digestion (Koike and Kobayashi, 2009). Next-generation sequencing (NGS) allows for the sequencing of thousands of unique DNA fragments from a single sample and can be used to quantify and assess microbial diversity in the rumen (Petri et al., 2013). It also allows for identifying microorganisms that cannot be cultured, as identification is based on DNA sequencing and does not require the bacteria to be grown in the laboratory. Therefore, application of NGS may provide important information on how different barley varieties influence cereal grain digestion and microbial diversity in the rumen.

2.0 LITERATURE REVIEW

2.1 Microbial Ecology in the Rumen

The ruminant host has a symbiotic relationship with ruminal microorganisms providing an anaerobic environment with a suitable pH range for their survival and growth as well as fermentable substrates from which volatile fatty acids are produced and utilized by the host for maintenance and growth (Zhou et al., 2009). The rumen is populated by a complex microbial ecosystem consisting of bacteria (10^{11} cells per ml), archaea (0.3 – 3% of the biomass), protozoa (10^5 to 10^7 cells per ml), and fungi (10^4 cells per ml) (Ozutsumi et al., 2005).

These ruminal microorganisms efficiently hydrolyze carbohydrates such as cellulose, hemicelluloses, pectin, starch and other polysaccharides within feed. The products of carbohydrate fermentation are volatile fatty acids (VFA; i.e., acetate, propionate, butyrate and branched chain VFAs), methane, carbon dioxide and microbial protein. The VFAs are absorbed through the rumen wall into the bloodstream, and are metabolized for energy and to meet the maintenance and tissue-building requirements of the host. In addition, dietary proteins are hydrolysed to amino acids and peptides, which are converted to ammonia and branch chain VFAs (Cotta and Russell., 1982).

Efficient microbial fermentation requires strict anaerobic conditions and a suitable rumen pH. Generally, the pH is between 5.8 and 6.8, but depends on the nature of the diet consumed. High grain diets can lead to a decline in ruminal pH due to the rapid production of VFA (Slyter**,** 1976). Fibre-digesting bacteria are inhibited at a pH less than 6.0 (Slyter, 1986; Roger et al., 1990), but some starch-digesting bacteria remain active even when rumen pH declines below 5.0 (McAllister et al., 1990). Ruminal microorganisms are also affected by diet as they do not

randomly colonize and digest feed particles. They have specific mechanisms to attach and adhere to feed particles to which they are more adept at digesting (Miron et al., 1990; Miron, 1991; Pell and Schofield, 1993; McAllister et al., 1994). Consequently, fibre-digesting bacteria adhere to fibre and starch-digesting bacteria adhere to starch. Ruminal fungi attach to both feed types and penetrate recalcitrant plant structures (McAllister et al., 1994). Protozoa are also involved in the digestion of both fibre and starch, adhering to the surface of plant cell walls and engulfing starch granules. (Bonhomme, 1990; McAllister et al., 1994). Thus, the rumen microbial ecosystem is dynamic and the abundance and diversity of microbiota is affected by multiple factors related to the host and feed ingested.

2.1.1 Ruminal bacteria and archaea

Rumen bacteria number from 10^{10} to 10^{11} bacteria cells per mL, and are responsible for the majority of fermentative activity in the rumen (Miron et al., 2001) as they have a variety of enzyme activities, including cellulases, xylanases, β-glucanases, pectinases, amylases, and proteases (Wang and McAllister, 2002). The bacteria population can be affected by feed type, animal type, and feeding program (Ogimoto and Imai, 1981; Stewart and Bryant, 1988), however feed type is the major factor that influences the composition of the bacterial population (Tajima et al., 2001). For example, Tajma et al., (2001) used real-time PCR to show that after transition of cattle from a hay to grain diet, the predominant bacteria evolved from those involved in the digestion of fibre to those involved in the digestion of starch.

The rumen bacteria can be classified on the basis of function based on their fibrolytic, amylolytic or proteolytic enzyme activities. The major known fibrolytic bacteria are *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Butyrivibrio fibrisolvens*,

Prevotella ruminicola, *Eubacterium cellulosolvens* and *Eubacterium ruminantium* (Stewart et al., 1997). The major amylolytic bacteria are *P. ruminicola*, *Ruminobacter amylophilus*, *B. fibrisolvens*, *Selenomonas ruminantium*, and *Streptococcus bovis*, which produce amylases to digest starch. The proteolytic bacteria; *R. amylophilus*, *P. ruminicola* and *B. fibrisolvens* digest feed protein in the rumen. For digestion of feed in the rumen, the rumen bacteria may have either synergistic or antagonistic relationships. For example, Scheifinger and Wolin, (1973) showed that *S. ruminantium* (a non-cellulolytic species) responded synergistically in co-culture with *F. succinogenes* (a cellulolytic species). In contrast, the growth of *R. flavefaciens* on cellulose was inhibited during co-culture with *R. albus* (Odenyo et al., 1994).

Based on sequencing of the microbial small subunit rRNA, it has been shown that *Archaea* account for approximately 0.3 to 3.3% of the combined 16S and 18S rRNA genes in the rumen (Janssen and Kirs, 2008). The domain *Archaea* includes the phyla *Crenarchaeota* and *Euryarchaeota*, with the latter accounting for rumen methanogens (Sirohi et al., 2010). There are 113 species of methanogens (Janssen and Kirs, 2008), but only 8 species have been cultured from the rumen (Patra et al., 2017), indicating that the ruminal environment is highly selective. Within the rumen, methanogens are found in free form and are also associated both internally and externally with protozoa (Sharp et al., 1998). They have a symbiotic relationship with protozoa, through interspecies hydrogen transfer. The rumen methanogens utilize hydrogen and carbon dioxide, which are formed during fermentation of carbohydrates to volatile fatty acids, to produce methane (Hook et al., 2010). Without the removal of hydrogen, proper rumen fermentation will be inhibited. Overall, methane production can account for approximately 2 to 12% of ingested feed energy (Johnson et al., 1995), highlighting the important role that methanogens have in ruminant production.

2.1.2 Ruminal Protozoa

Ciliated protozoa are the second most common microbe in the rumen; accounting for 30 - 40% of the total rumen microbial biomass (Van Soest, 1994). The ciliated protozoa are classified into two groups, the holotrichs and the entodiniomorphids, which are differentiated on the basis of morphological characteristics (Williams and Coleman, 1992). The holotrich protozoa range in size from 36 x 19 μm to 160 x 100 μm, are covered with cilia and are more motile than entodiniomorphid protozoa (Coleman, 1986). The entodiniomorphid protozoa can appear as flattened cylinders or spheres and are covered with a pellicle. They range in size from 24 x 15 μm to 205 x 123 μm (Coleman, 1986). The holotrichs are able to utilize soluble carbohydrates such as glucose, fructose, galactose, oligomers and non-structural polysaccharides such as starch (Orpin and Letcher, 1978; Coleman, 1986). The products of carbohydrate fermentation are D-and L-lactate, butyrate, acetate and propionate as well as hydrogen and carbon dioxide. Protozoa can also store polysaccharides in the form of glycogen and generate protozoal protein as a result of their growth. The holotrichs can also degrade cellulose and hemicellulose, but the rate of this degradation is very low compared to the entodiniomorphids (Williams and Goleman, 1985).

The entodiniomorphids also engulf starch granules and digest them through production of amylases (Coleman, 1986). All entodiniomorphid protozoa engulf and digest plant fibres through the production of a range of cellulases including α-glucosidase, β-glucosidase, and hemicellulases (Delfosse-Debusscher et al., 1979; Williams and Coleman, 1985). However, in general, rumen protozoa are not responsible for the same extent of feed digestion as are the rumen bacteria (Williams and Wither, 1993).

2.1.3 Rumen fungi

Rumen anaerobic fungi can be classified into six genera (Ho and Barr, 1995), which are *Neocallimastix* (Vavra and Joyon, 1966), *Piromyces* (Gold et al., 1988), *Caecamyces* (Gold et al., 1988), *Orpinomyes* (Barr et al., 1989), *Anaeromyces* (Breton et al., 1990), and *Cyllamyces* (Ozkose et al., 2001). These six genera are identified on the basis of morphological features, such as thallus morphology (rhizoidal vs. bulbous) and zoospore flagellation (monoflagellate vs. polyflagellate) (Ozkose et al., 2001). Ruminal fungi make up only 5-10% of total rumen microbial biomass. Even though they account for a realtively low proportion of rumen microbial biomass, fungi may be important for the digestion of poor quality forages (Ho and Abdullah, 1999). Ruminal fungi can produce hydrolytic enzymes, including cellulases and hemicellulases (xylanase) and the ability of their rhizoids to penetrate plant cell walls enables them to effectively degrade this mixture of complex carboydrates (Lowe et al., 1987).

2.2 Structure and composition of barley grain

The major parts of the barley grain include the hull, the endosperm, and the germ. The hull is the outer layer of the barley grain and accounts for 10-13 % of the kernel weight (Evers and Millar, 2002). The high fibre content of the hull can protect the interior endosperm from attack by insects, fungi and bacteria (Briggs, 1998; Olkku et al., 2005). Generally, the hull consists of two structures known as the lemma and palea, which cover the ventral and dorsal side of the grain, respectively. In barley, the hull is tightly attached to the pericarp (Olkku et al., 2005).

The endosperm consists of the starchy endosperm and a surrounding aleurone layer. It contains carbohydrates, proteins and small amounts of vitamins and minerals. The starchy

endosperm forms the largest morphological part of the barley grain kernel comprising approximately 75% of its weight (Evers and Millar, 2002). The endosperm provides nourishment for the growing embryo during germination. The aleurone layer surrounds the starchy endosperm, and consists mainly of arabinoxylan (Fincher and Stone, 1986), protein, lipids, vitamins and minerals (Evers and Millar, 2002). The germ or embryo is the smallest part of the grain, but has the highest concentration of protein, oils, and vitamins to aide in the germination of the seed (Bhavna and Carol., 1981; Dawei et al., 2014).

2.2.1 Barley hull and pericarp

The hull (outer lemma and inner palea) is firmly adherent to the pericarp in barley grain and serves to protect the endosperm from microbial attack in the field (Broderick and Vogel, 1977) (Figure 2.1). The hull is mostly composed of lignin, pentosans, mannan, uronic acids, hemicellulose, and cellulose (Briggs, 1998; Olkku et al., 2005). The pericarp also comprises an outer protective tissue layer (Figure 2.1). The pericarp is under the hull and consists of highly compressed cell material bound by a thin outer cuticle. The chemical composition of the hull and pericarp can be affected by kernel size, variety, growing conditions, crop genetics and agronomic management practices (Evers *et al*., 1999).

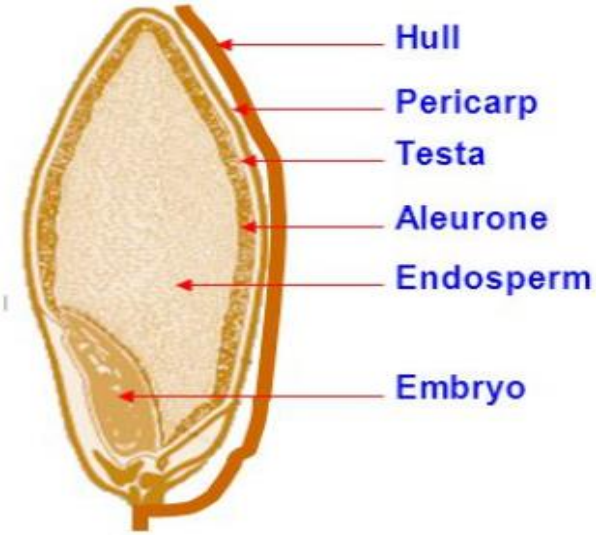


Figure 2.1. Diagram of the barley grain, showing the different tissues presented by Elsayed (2014).

2.2.2 Protein matrix

The protein content of barley grain ranges from 7.5 to 17% on a dry matter basis. However, this protein concentration is influenced by the availability of nitrogen, which when increased through the use of fertilizer has been shown to increase the protein content of barley from 7.87 to 12.94% (Kirkman et al., 1982). The proteins in barley grain can be classified into three classes based on their solubility: globulins (salt-soluble), prolamins (alcohol-soluble), and albumins (water-soluble) (Osborne, 1924; Gubatz and Shewry, 2011). The storage globulins account for 10-20% of the total protein content of barley grain (Lásztity, 1984), and are mainly located within aleurone cells and the embryo (Yupsanis et al., 1990). The barley non-storage proteins are also found in the aleurone and embryo, and account for 15-30% of the total protein with albumins and globulins as the major representatives. The prolamins are located only in the starchy endosperm (Yupsanis et al., 1990) and are the major storage proteins, accounting for 35-55% of the protein content of barley grain. However, the aleurone and embryo are much more nutritionally rich proteins compared with the starch endosperm because the albumin and globulin contain about 5-7% lysine as compared to less than 1% lysine in prolamins (Shewry et al., 1984).

2.2.3 Starch granules

In mature barley grain, starch granules are embedded in a protein matrix. Barley endosperm matures through three developmental steps, including early development, differentiation, and maturation (Sabelli and Larkins, 2009). During the maturation step, the barley endosperm consists of five tissues, the interior starch-filled endosperm, the sub-aleurone layer, the aleurone layer, the basal endosperm transfer layer, and embryo surrounding region (Olsen et al., 1999). The mature barley endosperm accounts for 75 – 80% of the total weight of

the kernel and consists of starch (85-89%), protein (10%), β-glucan and small amounts of vitamin and minerals (MacGregor et al., 1993). Starch is composed of amylopectin and amylose glucan polymers. Amylose is composed of alpha-(1, 4)-glycosidic linkages, whereas amylopectin has a higher molecular weight with alpha-(1, 4)-glycosidic and alpha-(1, 6)-glycosidic branched linkages (Pomeranz, 1985). Generally, amylopectin molecules are about 10 times larger than amylose molecules. The amylopectin to amylose ratio in the endosperm can be affected by barley variety, and are described as normal, waxy (amylopectin up to 100%), and high-amylose (amylose up to 70%) depending on the ratio present.

2.2.4 Embryo

The embryo plays a critical role in the germination of the kernel. It contains up to 34% protein, nucleic acids, lipids (14-17%), ash and water. The embryo consists of a number of acrospires, which include coleoptile, leaf primordia, apical meristem a nodal region between the root and the shoot, and a primary root covered by the coleorhiza (Anna et al., 1966). The embryo is separated from the endosperm upon germination by the scutellum, which is a modified cotyledon.

2.3 Barley grain varieties

The major nutritional constituents of barley grain are starch, dietary fibre and protein, which account for approximately 60, 20 and 12% of dry matter, respectively. Barley grain varieties can be genetically classified as spring or winter types. Barley is further categorized as hulled or hulless, and as being two- or six-row (Cereal Foods World, 2005). In addition, barley varieties can be classified based on their chemical composition being described based on their starch characteristics as normal, waxy or high amylose starch or on their level of lysine (i.e., high

lysine) or β-glucan content (Sullivan et al., 2013). In Canada, there are some public institutions that are breeding for improved barley varieties, including Agriculture and Agri-Food Canada (AAFC), Alberta's Field Crop Development Centre (FCDC), the Crop Development Centre (CDC) at the University of Saskatchewan (U of S), the National Research Council (NRC), the University of Alberta (U of M) and the University of Manitoba (U of M). Over the last 41 years, a total of 32 barley varieties have been released, 23 of which are feed varieties with the remainder being varieties that are targeted for malt or as food. Barley grain is predominantly used for feed (70%), malt (20%) food (5%) and other undefined purposes (5%) (Wang, 2005). Improving the quality and yield of feed barley is an important objective for the feed industry.

2.3.1 Differences in chemical composition among barley varieties

The composition of barley grain can vary considerably depending on the variety and growing conditions (Nilan and Ullrich, 1993). In addition, the chemical composition of barley can vary seasonally (Bradshaw et al., 1992). Starch, fibre and protein content range from 45.9 to 62.8%, 17.3 to 32.1% (Ovenell-Roy et al., 1998a; Khorasani, 2000), and 7.5 to 16.2 %, respectively (LaFrance and Watts, 1986; Khorasani, 2000). Two-row varieties generally produce plumper kernels and higher test weights with higher starch content than six-row varieties (Legge et al., 2012). Hulless barley varieties have lower fiber, but higher protein and energy levels than hulled barley varieties (Bhatty, 1986). Similarly, feed varieties generally have higher protein content as compared to non-feed varieties (Popova et al., 2013). It is possible that differences in the chemical composition among barley varieties alter the types and density of rumen microbes that access and digest barley in the rumen (Reynolds et al., 1992).

2.3.1.1 Hulled and hulless barley varieties

Hulled barley has an outer hull attached to the kernel, a structure that is lacking in hulless barley varieties. As of 2014, there were 20 varieties of two-row - spring - hulless barley, eight varieties of six-row - spring - hulless barley and four varieties of two-row - waxy -hulless barley in the National Registry (Sarah, 2015). Nutritionally, hulless barley has higher contents of starch and protein, and lower fibre than hulled barley varieties (Nikkhah, 2012), a compositional difference that reflects the absence of the hull (Yang et al., 1997). As compared to hulled barley varieties, hulless barley also contains more essential amino acids, including arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine (National Research Council, 1988). These differences in nutritional composition can affect digestion in the rumen. Generally, hulless barley varieties have higher energy values and better nutrient availability due to the fibre content reduced and increased starch content compared to hulled varieties (Zinn et al., 1996). However, cattle fed hulless barley exhibited lower feed intake than those fed hulled barley due to a reduction in rumen pH, a reflection of the excessive accumulation of acids in the rumen (Zinn et al., 1996). Hulless barley varieties also have a lower yield than hulled varieties, resulting in the production of less digestible DM per ha (Sarah, 2015).

2.3.1.2 Two-row and six -row barley

Two-row and six-row barley can be distinguished by the number of rows of kernels within the seed head. Two-row barley has 2 rows of seeds on each spike and six-row has 6 rows of seeds on each spike (Cereal Foods World, 2005). In general, six-row barley tends to be slightly shorter and thinner as compared to two-row barley (Gebhardt et al., 1993). Although the plumpness of six-row barley has been generally increasing, two-row barleys usually produce

plumper kernels than six-row barley. The plumpness of two-row barley varieties is usually a reflection of the higher starch content within the kernel. Therefore, two-row barley varieties contain more starch and fat, and are lower in Ca, ADF, and β-glucans as compared to six-row barley varieties (Richard and Lee, 1995). In addition, six-row barley varieties generally have higher protein content than two-row barley varieties (Goldammer, 2008). In the rumen, the total tract NDF digestibility of two-row barley is generally greater than six-row barley varieties (Ovenell-Roy, 1998b).

2.3.2 Starch type

In general, starch accounts for 50 to 60 % of the dry matter of barley grain (Nocek and Tamminga, 1991). The starch components of barley grain mainly consists of the two water-insoluble homoglucans, amylose and amylopectin (Delcour and Hoseney, 2010; Perez and Bertoft, 2010). Generally, barley starch consists of 25% amylose and 75% amylopectin, which is defined as a normal starch type (Delcour and Hoseney, 2010). Waxy starch type barley has higher amylopectin content (95 -100%), and amylose starch type varieties contain up to 70% amylose. These amylose and amylopectin ratios may have an impact on starch digestibility in the rumen. The low amylose barley varieties had a higher effective degradability (0.148/h) as compared to normal amylose (0.115/h) and high amylose varieties (0.102/h) in an *in vitro* ruminal batch culture study (Stevnebø et al., 2009).

2.3.2.1 Amylose

Amylose consists of a linear structure with α-(1,4)- linkages between α-D-glucose residues (Delcour and Hoseney, 2010) with a minor degree of branching as a result of α-(1,6)-linkages. Amylose contains 500 -6,000 glucose units within each chain, resulting in a lower

molecular weight than amylopectin. Generally, amylose is degraded by α-amylases, which release maltose, maltotriose and occasionally small amounts of free glucose. β-amylases specifically release maltose units from the linear chain with members of the amylolytic bacterial population in the rumen collectively producing all of these amylases (Chesson, 1997; Huntington, 1997). However, amylose is more resistant to degradation in the rumen than amylopectin, due to hydrogen bonding being stronger in amylose chains with a higher degree of polymerization as compared to amylopectin (Hibberd et al., 1982; Cone and Wolters, 1990). In addition amylose is often complexed with lipids (Tufvesson et al., 2001) and proteins, which can also impact, starch digestibility (Brennan et al., 1996).

2.3.2.2 Amylopectin (Waxy starch)

Amylopectin is a heavily and highly branched polysaccharide consisting of α-(1–4)-linked D-glucopyranosyl chains, which are interconnected via α-(1–6)-glycosidic linkages (Zobel, 1988). The glucose chains are shorter than in amylose, but more branched, resulting in a higher molecular weight than amylose (Bulèon et al., 1998). Generally, barley with a high amylopectin starch type is referred to as "Waxy starch", which contains 95 to 100% amylopectin. Waxy starch types are more readily digested due to their greater susceptibility to enzyme hydrolysis than high amylose barley types. However, because waxy starch varieties have a higher β-glucan and protein content (Tester and Morrison, 1993), the starch content of these varieties is generally lower than non-waxy types. Furthermore, increased concentrations of these other cellular components may influence the digestibility of starch in the rumen. For example, Foley et al. (2006) showed that total tract apparent digestibility of dry matter and organic matter was lower for a wax barley variety as compared to a normal barley variety. However, waxy barley enhanced the incorporation of NH_3 into ruminal bacteria, and also reduced urinary N

losses to a greater extent than normal barley. It has been reported that normal and waxy barley varieties do not differ in their effects on pH or VFA concentrations in the rumen (Hristov et al., 2002). Owing to the complexity of amylolytic bacterial populations in the rumen, differences in the effective digestibility of starch among starch types is generally small with major differences in ruminal starch digestibility being more related to the structural components within the endosperm rather than the nature of the starch itself (McAllister et al., 1993).

2.4 Role of rumen microbes in grain digestion

2.4.1 Attachment – biofilm formation

Most ruminant feeds have protective barriers that preclude colonization and penetration by rumen microorganisms. These structures include the cuticular layer in forages and the pericarp in cereal grains, which can also contain antimicrobial compounds such as condensed tannins and phenolic acids (McAllister et al., 1990; Forsberg and Cheng, 1992). Although the cuticle and epicuticular waxes are resistant to rumen microorganisms and digestive enzymes, mastication and processing disrupts these resistant barriers and improves ruminal digestion (Akin, 1989). Physical and chemical processing of feeds can increase the extent and rate of feed digestion by the enzymes that are produced by ruminal microorganisms (Beauchemin et al., 1993). Rumen microorganisms have specific mechanisms to attack and colonize feeds, and produce enzymes to digest chemically and structurally complex feed particles in the rumen (McAllister et al., 1994). In most instances, ruminal bacteria and fungi are responsible for the degradation of 80% of the feed particles in the rumen, with the remainder of degradative activity (20%) residing with protozoa (Dijkstra and Tamminga, 1995).

Ruminal bacteria can be classified into three distinct subpopulations based on their location within the rumen, as those adhering to the rumen wall, those in rumen fluid, and those adhering to feed particles (McAllister et al. 1994; Dehority, 2003). Feed particle associated bacteria can further be classified into loosely and firmly associated populations (Larue et al., 2005). The firmly feed-associated populations are responsible for approximately 80-90% of fiber digestion, 75% of protein digestion, and 70% of starch digestion (Brock et al., 1982; Minato et al., 1993; McAllister et al., 1994).

Ruminal bacteria are able to gain access to the interior of feed particles where they attach and form biofilms and digest, and produce multiple enzymes (Cheng et al., 1984; Craig et al., 1987). The attachment of ruminal bacteria to feed particles is accomplished through specific mechanisms that require adhesins and receptors as well as non-specific mechanisms (Pell and Schofield, 1993). For example, Stack and Cotta (1986) showed that 3-phenylpropanoic acid improves the adhesion of *R. albus* to cellulose, but has no effect on the adhesion of *R. flavefaciens* or *B. fibrisolvens*.

In contrast to other microorganisms in the rumen, fungi physically penetrate and enzymatically digest undamaged plant material (Ho et al., 1988; Orpin and Joblin, 1997). Ruminal fungi produce most of the hydrolytic enzymes required to break down the major components of plant biomass. These enzymes include cellulases, hemicellulases, pectinlyases, amylases, and proteases. Also, ruminal fungi produce phenolic esterases (p-coumaroyl and feruloyl) that can break the cross linkages between hemicelluloses and lignin, increasing access to hemicellulose (Borneman, et al., 1990). The fungi hyphae are capable of penetrating plant tissue which may provide access points for bacteria to enter and digest the more readily digestible internal components of plant cells (Ho et al., 1988).

Ruminal protozoa can be classified into compartments in a manner similar to ruminal bacteria, as they can be distinguished as being part of the liquid, solids or epimural associated populations. The ruminal protozoa usually adhere to large feed particles and engulf starch granules (Bonhomme, 1990).

The rate of feed colonization is generally faster for bacteria than for protozoa and fungi. Protozoa and fungi slowly colonize feed particles, a phenomenon which is a reflection of their generation times, which is 5 to 14 h for protozoa (Williams and Coleman, 1988) and 24 to 30 h for ruminal fungi (Bauchop, 1981; Joblin, 1981). In comparison, some bacteria have generation times of less than one hour allowing for rapid colonization and proliferation.

2.4.2 Role of amylolytic microbes

More than 90% of the starch in cereal grains can be digested in the rumen, with the rate of starch digestion being influenced by grain type, the method of processing and the nature of the rumen microbiome. The ruminal bacteria are responsible for the majority of starch digestion in the rumen. The numbers of amylolytic bacteria within the microbiome increase when cattle are fed diets with increasing levels of starch. Starch is an important energy source in the diet as it is highly fermentable and results in high yields of VFA to support meat and milk production. Although starch represents a valuable energy source for ruminants, it also can be associated with metabolic disorders such as acidosis (Gressley et al., 2011), low–fat milk syndrome and liver abscesses (Owens et al., 1998). This is due to the high levels of lactic acid and low pH that can be associated with these diets.

Ruminal protozoa (Holotrichs and Entodiniomorphs) with high amylase activity are also involved in starch digestion as they engulf starch granules (Williams and Coleman, 1992).

Under some conditions, protozoa can account for up to 50 % of the starch digestion in the rumen (Jouany and Ushida, 1999). Protozoa also are predators of amylolytic bacteria, which can lead to a reduction in the numbers of bacteria in the rumen and a corresponding reduction in the rate of ruminal starch digestion.

2.4.3 Role of fibrolytic microbes

Ruminants are able to ferment and utilize fibre due to the presence of fibrolytic microbes in the rumen. Fibrolytic microbes include bacteria, protozoa and fungi, which produce multiple enzymes such as cellulases, xylanases, β-glucanases, pectinases and esterases (Ali et al., 1995; Bayer et al., 2006; Dashtban et al., 2010).

The fibrolytic bacteria are responsible for the majority of fibre degradation in the rumen because of their much larger biomass and associated enzyme activity. Furthermore, fibre digestion can be improved by nutritive interactions and cross feeding of fermentation products between fibrolytic and non-fibrolytic species (Flint, 1997). For example, the combination of fibrolytic species *F. succinogenes*, *R. flavefaciens* or *R. albus* with non-fibrolytic *Treponema* or *Butyrivibrio* species accelerates cellulose digestion (Cheng et al., 1991).

Protozoa are also produce enzymes involved in the digestion of cellulose (Jouany and Senaud, 1979). *In vitro* studies have suggested that 19–28% of total cellulase activity is attributable to protozoa (Gijzen et al., 1988). However, protozoa can cause a decrease in bacterial cellulolytic activity due to predation on fibrolytic bacteria in the rumen (Kurihara et al., 1968).

Fungi are also involved in the digestion of cellulose as they possess cellulase and hemicellulase activities (Orpin and Joblin 1988). Joblin (1989) showed that ruminal fungi degraded 37–50% of barley straw, whereas rumen bacteria digested only 14–25%. Therefore, the rumen fungi play an

important role in fiber digestion, as they are able to penetrate deeply into lignified plant tissues that are normally inaccessible to bacteria (Bauchop, 1981; Akin 1986).

2.4.4 Role of proteolytic microbes

Dietary protein is rapidly degraded by microbial proteases to peptides and amino acids by rumen bacteria, protozoa and fungi (Hobson and Wallace, 1982 and Wallace and Cotta, 1988). Rumen bacteria are primarily responsible for the degradation of protein (Nugent and Mangan 1981; Brock et al., 1982), as their proteinase activity is 6 to 10 times higher than protozoa. This activity has also been reported in *S. bovis* and *Provotella albensis* (Sales-Duval et al., 2002). Amino acid and peptide fermenting species such as *Clostridium aminophilum*, *Clostidium. sticklandii* and *Peptostreptococcus anaerobius* also play a role in ruminal N metabolism (Chen and Russell, 1989; Russell et al., 1988). Protozoa are also capably of utilizing both bacterial and dietary protein. The bacterial proteins are degraded within protozoa to amino acids (Coleman, 1975). Rumen fungi also have a high proteolytic activity; especially Neocallimastix *frontalis* which releases significant quantities of proteinases during the digestion of plant cell walls.

2.5 Barley grain processing

For optimal utilization, barley grain must be physically processed before being fed to ruminants as whole barley grain is not effectively digested in the rumen (Nordin and Campling, 1976; Valentine and Wickes, 1980) owing to the recalcitrant nature of the surrounding pericarp (Dehghan-banadaky et al., 2007). Physical processing of barley grain provides ruminal microorganisms with access to the internal structures of the kernel enabling enhanced enzymatic attack to increase the rate and extent of ruminal protein, fibre and starch degradation (Mathison,

1996). Processing methods can be described as either being cold or hot physical processing (Zinn, 1993; Yang et al., 2000). Cold physical processing methods include grinding, dry rolling, and tempering.

Grinding is a simple process that reduces the particle size of grain, increasing the surface area available for microbial attachment, and the rate of starch degradation in the rumen (Galyean et al., 1981). However, grinding methods can cause shattering and the formation of fine particles, which can be rapidly fermented in the rumen, giving rise to subclinical and clinical acidosis and a reduction in dry mater intake (Mathison, 1996) as well as adverse impacts on growth and health.

Dry rolling is one of the most common methods of processing grains for ruminants. This method generates larger more uniform particles than grinding. Compared to whole grain, dry rolling increased the organic matter digestibility of barley from 52.5 to 85.2% (Toland, 1976). Similarly, rolled barley fed to beef steers resulted in a far higher ADG (1.37 kg /day) than whole barley (1.30 kg/d) (Mathison et al., 1991).

Tempering barley is a process where water is added to grain to increase the moisture to 18-20% prior to rolling, a practice that reduces the amount of fine particles generated as compared to dry rolling (Yang et al., 1996; Dehghan-banadaky et al., 2007). Research with dairy cattle has shown that milk production was increased when temper rolled barley was fed as compared as to dry rolled barley (Christen et al., 1996). In addition, compared to dry rolled barley grain, tempered barley grain improved milk yield by 5%, feed efficiency by 10%, and digestibility of DM, NDF, ADF, starch by, 6, 15, 12 and 4%, respectively (Christen et al., 1996). Hot physical processing methods use heat, moisture, and pressure during processing. The application of heat and moisture during processing gelatinizes starch and may increase its

degradation in the rumen (Waldo, 1973). The most common method of hot physical processing is steam rolling. A benefit of this method is that there is a reduction in the amount of fine particles as compared to dry rolled barley.

Steam flaking is also a hot physical processing method, where a high moisture treatment disrupts the protein matrix in the peripheral endosperm improving protein and starch digestibility. Steam flaking of barley improved starch digestion in cattle as compared to dry rolling (Owens and Zinn, 2005). Similarly, steers fed steam flaked barley had higher rates of gain (0.10 kg/day) and improved feed intake (0.86 kg/day) when compared to dry rolled barley (Hale et al., 1966). However, no differences in average daily gain or feed efficiency were observed in finishing cattle fed dry rolled vs steam flaked barley (Grimson et al., 1987).

2.6 Impact on site of barley grain digestion in ruminant digestive tract

High concentrate diets that contain processed barley grain are usually rapidly digested in the rumen and can have a significant impact on microbial activity, rumen function, and animal productivity and health (Nagaraja and Lechtenberg, 2007; Petri et al., 2013). For optimal fibre digestion, ruminal pH must generally be above 5.8 to 6.0 (De Veth and Kolver, 1999; Van Soest, 1994). However, high grain diets increase the concentration of volatile fatty acids (VFAs) and lactic acid in the rumen, which can reduce ruminal pH below this optimal level (Nocek, 1997; Klieve et al., 2003). When rumen pH falls below 6, rumen microbial populations shift from primarly a fibrolytic to a more amylolytic population (Tajima et al., 2001). For example, adhesion of *F. succinogenes* to cellulose was decreased when ruminal pH fell below 6.0 (Roger et al., 1990). In another study, the fibrolytic activity of *R. albus* decreased at ruminal pHs below 5.0 (Morris, 1988).

At ruminal pHs below 5.2, numbers of many of the gram negative bacteria become reduced, including lactate utilizing bacteria, such as *Megasphaera elsdenii* and *S. ruminantium* (Nagaraja and Titgemeyer, 2007; Hernández et al., 2014). In contrast, gram positive, lactate producing bacteria such as *S. bovis* and *Lactobacillus* increase in numbers (Dawson et al., 1997; Allen et al., 2006). Some of these lactate producing bacteria can produce D or L–lactic acid isomers. The D and L – lactic acid is absorbed across the rumen wall and depress blood pH even though L-lactate is rapidly metabolized in the liver. In contrast, D-lactate is not as rapidly metabolized in the liver and can lead to systemic metabolic acidosis (Bolton and Pass, 1988). When rumen pH reaches 4.5, *Lactobacillus* spp. predominate resulting in the accumulation of lactate within the rumen and a further drop in ruminal pH. Ruminal acidosis can be classified into two types, acute or subacute. In general, acute acidosis is defined as when ruminal pH drops below 5.0 and lactic acid accumulates in ruminal fluid. Sub-acute ruminal acidosis generally occurs when ruminal pH ranges from 5.0 to 5.5 (Galyean and Rivera, 2003). Severe ruminal acidiosis can also precipitate other disorders including laminitis, lameness and liver abscesses (Owens et al., 1998).

2.7 Rumen digestibility methodology

2.7.1 *In situ* / *in vitro* techniques for measuring digestibility in the rumen

The digestibility of ruminant feeds can be estimated using a number of different techniques. These techniques can be classified as biological methods, which involve the direct use of the animals, by digesting feeds in the rumen such as the *in situ* method. Other techniques are laboratory based which do not require the direct use of animals and simulate the rumen environment and digestion process using rumen fluid collected from animals or artificial enzymatic mixtures that model digestion within the intestinal tract.

The *in situ* or nylon bag technique has been used for many years to provide estimates of both the rate and extent of disappearance of feeds both in the rumen and lower intestinal tracts (Mirzaei-Aghsaghali et al., 2008; de Boer et al., 1987). *In situ* nylon bag techniques are quite useful for evaluating kinetic aspects of digestion in ruminants. Through the use of multiple incubation times, the rates of nutrient digestion can be estimated. This technique is also useful for comparing the effect of the host animal on ruminal digestibility (Marinucci et al., 1992). Ellis et al. (1994) suggested that the *in situ* method is preferable to *in vitro* techniques because it more closely simulates the rumen environment. However, the result of ruminal digestibility with this technique can be affected by the feeding frequency of the animal and also the pore size of nylon bags (Khan et al., 2003).

The *in vitro* technique (Tilley and Terry, 1963) is also commonly used to estimate the digestibility of feedstuffs for ruminants (Uden, 1992; Fahey and Hussein, 1999). *In vitro* digestibility techniques provide a quick and inexpensive, analysis of digestibility for a large-scale evaluation of ruminant feeds (Tilley and Terry, 1963). However, this technique compared to the *in situ* method, may overestimate ruminal digestibility of feed particles. Di Marco et al. (2005) showed that the DM digestibility of corn silages after 24 h of *in vitro* incubation using rumen inoculate from Holstein cows was approximately 15% higher than *in situ* and *in vivo* techniques.

2.8 Rumen molecular microbiology

2.8.1 Molecular techniques for ecological characterization in the rumen

The ruminant host has a complex symbiotic relationship with the rumen microbiome. The characterization and identification of the members of the rumen microbiome is pivotal to

developing an understanding of host-microbiota interactions and their impact on production and host health. Previously, ecological research on the rumen was mainly conducted using traditional culture-based techniques for identification and characterization of rumen microbial populations. However, culture-based techniques can only study the cultivable microbes that will grow on defined media (Krause and Russell, 1996). Based on 16S rRNA-based analysis, it has been suggested that 300-400 different bacterial species are present in the rumen (Edward et al., 2004; Yu et al., 2006). Culture-based methods generally recover a small portion of the diversity from environmental samples (Mason et al., 2012). However, advanced molecular techniques, are culture-independent as they are based on the isolation of nucleic acids (DNA and RNA) from microbial environments. This approach can reveal both structural and functional information as well as characterize the diversity and abundance of the members within the rumen microbial community (Vartoukian et al., 2010; Mason et al., 2012). Next generation sequencing (NGS) techniques (Nowrousian, 2010) allow large-scale analysis of rumen microbial communities through the sequencing of millions of gene sequences from individual samples.

2.8.2 Next generation sequencing technologies

The first widely adopted DNA sequencing technology was Sanger sequencing (Sanger et al., 1977). This method was based on the DNA polymerase-dependent synthesis of a complementary DNA strand. For 30 years, the process speed, read length, throughput and cost of Sanger sequencing was reduced. However, it was still limited to a single type of DNA and did not prove useful for community analyses. Within the last 10 years, new types of mass sequencing technologies have been developed, collectively termed as next generation sequencing. These NGS techniques have been applied in variety areas, such as diagnostics, drug discovery, biomarker discovery, personalized medicine, and agricultural research. For bacterial and archaeal

community studies, NGS has typically involved sequencing of variable regions of the small-subunit ribosomal RNA gene (16S rRNA gene) to investigate the molecular phylogeny of samples. More recently, the rumen bacterial population of cattle has been extensively studied (Bath et al., 2013; Myer et al., 2015) by sequencing 16S rRNA regions (Jami et al., 2014; McCann et al., 2014).

Each of the NGS sequencing platforms has different chemistries and detection techniques, so they all have their strengths and weaknesses The five most common NGS techniques are Roche 454, Illumina, SOLiD (Valouev et al., 2008), Ion Torrent and Pacific Biosciences (PacBio) (Eid et al., 2009). Roche 454 (Margulies et al., 2005) was the first NGS technology developed in 2005. At this time, the Roche 454 had longer reads then other techniques, but relatively low throughput and a high reagent cost. High error rates in homopolymer repeats also limited the value of this technology (Metzker, 2010). The second generation of NGS technology was developed by Illumina by Solexa in 2007. Illumina had a higher throughput and the lowest per-base cost, but sample loading was technically challenging. In 2007, the third NGS technology was released as Oligo Ligation Detection (SOLiD) by Life Technologies. This NGS technology had the second highest throughput and lower error rates (99.94% accuracy), but the shortest read length with the longest run time. Ion Torrent technology was released by Jonathan Rothberg in 2010. This technology had a shorter run time and lower cost, but was plagued by high error rates in the form of homopolymers. The latest commercial NGS technology has been developed by PacBio, which produces long read lengths, but at a high cost with moderate error rates (~14%).

2.8.3 454 pyrosequencing

In 2005, Roche 454 was the first NGS platform to achieve a high-throughput, large scale, commercial sequencing technique (Roche Applied Science, 2008). This pyrosequencing technology enabled at variety of applications because of its long read length and high level of accuracy and precision. Roche 454 sequencing comprises three primary steps; 1) DNA sample preparation, 2) A proprietary process to load the DNA sample onto beads and 3) Sequencing by synthesis. The first sample preparation step is to break the "double-helix" DNA ladder into shorter double-stranded fragments of approximately 400 to 600 base pairs and then attach adapters to the DNA fragments. Finally, the double-stranded DNA fragments are separated into single strands. In the second step the DNA is loaded onto beads. This step involves the process of emulsion-based clonal amplification, or emPCR, where the DNA library fragments are attached to micron-sized beads. Each bead carries a single sstDNA library fragment. The bead-bound library is emulsified with amplification reagents in a water-oil mixture. Each bead is separately captured within its own microreactor for PCR amplification. Amplification is performed in bulk, resulting in bead-immobilized, clonally amplified DNA fragments that are specific to each bead. The last processing step is sequencing by synthesis to generate sequence data. Starting at one end of the DNA fragment, DNA polymerase enzyme sequentially adds a single nucleotide that is the match of the nucleotide on the single strand. Nucleotides are paired one by one as the enzyme moves down the single stranded fragment to extend the double-helix ladder structure. Nucleotide sequences generate an observable light signal, which is recorded by a camera.

2.9 Defining microbial ecology using sequencing of 16Sr RNA

Over the past decade, characterization of the complex ruminal microbial communities has dramatically advanced as a result of Next Generation Sequencing (NGS) technology, such as 454 and Illumina. These NGS methods are based on molecular phylogenies of the small-subunit ribosomal RNA gene (16S rRNA gene). The 16S rRNA gene is one of the most conserved genes within bacterial genomes (Janda and Abbott, 2007), and contains conserved regions as well as nine hypervariable regions (V1-V9) (Figure 2.2). Primers targeting the conserved regions are used to PCR-amplify 16S rRNA sequences from bacterial communities. These amplicons can then be sequenced and the variable regions within the primers are compared to databases for classification and identification of bacteria in the samples.

The full length 16S rRNA gene has been used to classify isolates of bacteria based on sequence similarity, with genera identified at 90% similarity and species typically at 97% similarity. The relatively short read length of 454 (400-450bp) and Illumina (150-300 bp) technologies however, introduce challenges to taxonomic classification of bacteria from mixed populations. Because the reads do not cover the entire 16S rRNA gene, primers targeting specific variable regions are used. However, amplification of 16S rRNA gene with different hypervariable regions may result in different quantitative assessment of bacterial communities (Pinto and Raskin, 2012; Pitta et al., 2014). Because the read length of 454 sequencing approaches 400 bp, most bioinformatics studies with 454 sequencing span the V1-V3 regions as studies have shown that this region results in improved taxonomic resolution (Hamp et al., 2009; Kim et al., 2011a). For example, the V1-V3 hypervariable region had more informative fingerprinting microbial profiles with thesolid fraction of rumen contents from cattle as compared to V4-V5 and V6-V8 hypervariable regions (Pitta et al., 2014). In addition, a higher

abundance of *Bacteroidetes* with V1-V3 region in both solid and liquid fraction compared to V4-V5 and V6-V8 hypervariable regions have been described (Fernado et al., 2010; Pitta et al., 2014). Furthermore, coverage of *Prevotella* and other *Bacteroidetes* lineages were greater with V1-V3 region compared to V4-V5 and V6-V8 hyper-variable regions (Pitta et al., 2014). The abundance of lineages of *Firmicutes* (*Coprococcus*, *Lactonifactor*, *Sporobacter*) and *Fibrobacteres* (*Fibrobacter*) were also well represented with V1-V3 and V4-V5 regions compared to V6-V8 (Pitta et al., 2014). Therefore, the V1-V3 region offers more information on different bacterial phyla and covers more diverse bacterial lineages for ruminal bacteria than other regions. The coverage of the observed number of sequences, diversity and species richness with V1-V3 region has also been shown to be higher than with other regions (Fouts et al., 2012; Petri et al., 2013a).

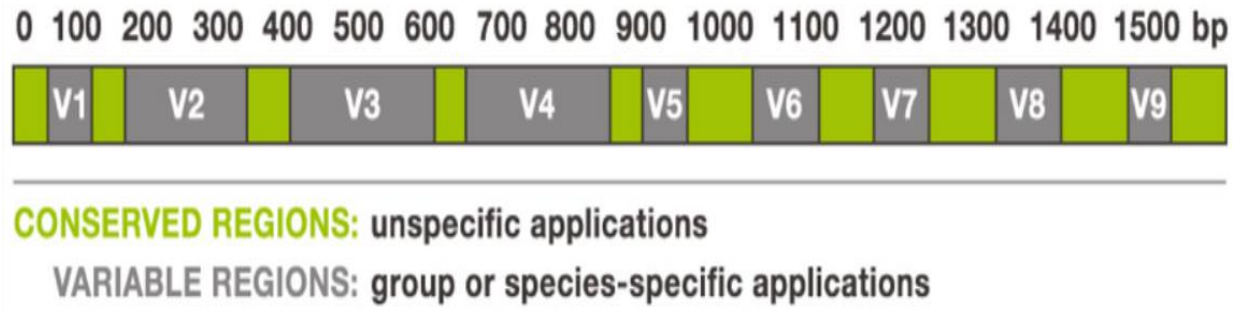


Figure 2.2. Sequence conservation and variability of 16S ribosomal RNA gene from Ward et al. (2012).

2.10 Bioinformatic analysis

Bioinformatics combines the fields of computer science, statistics, and mathematics to analyze large biological (eg. 16S rRNA gene) sequence data sets generated from next generation sequencing. Analysis of microbial communities requires the use of bioinformatics tools to efficiently and reproducibly process the large amount of data generated from amplicon based analysis using 16S rRNA gene sequences so as to derive taxonomic classifications. There are various bioinformatics tools to analyse16S rRNA gene sequencing data including QIIME (Quantitative Insights into Microbial Ecology) (Caporaso et al., 2010), Mothur (Schloss et al., 2009), MG-RAST (Metagenomics – Rapid Annotation using Subsystems Technology) (Meyer et al., 2008), Genboree (Riehle et al., 2012), EzTaxon (Kim et al., 2012), Pheonix2 (Soh et al., 2013), MEGAN (Mitra et al., 2011), CloVR-16S (Angiuoli et al., 2011), and the RDPipeline (Ribosomal Database Project Pipeline) (Cole et al., 2014). Generally, QIIME, Mothur and MGRAST are commonly used in the analysis of 16S rRNA sequence data. The three reference databases, RDP, SILVA, and Greengenes are used as reference databases with a 97% similarity being used to cluster reads for taxonomic classification. However, QIIME and Mothur are more powerful than MG-RAST because their statistical capabilities are more robust (Plummer et al., 2015). QIIME is capable of analyzing very large datasets in a short period and the command language is more user friendly than either Mothur or MG-RAST.

2.11 Using QIIME to analyze 16S rRNA sequences

QIIME is an open-source bioinformatics software package that is often used for the analysis of NGS sequences from microbial communities. The QIIME software was designed to analyze high-throughput sequences and to provide statistical analyses of data and visualization of

results (Kuczynski et al., 2012). QIIME can be used for data preprocessing, measuring alpha and beta diversity, and assessing the taxonomic composition of sequences.

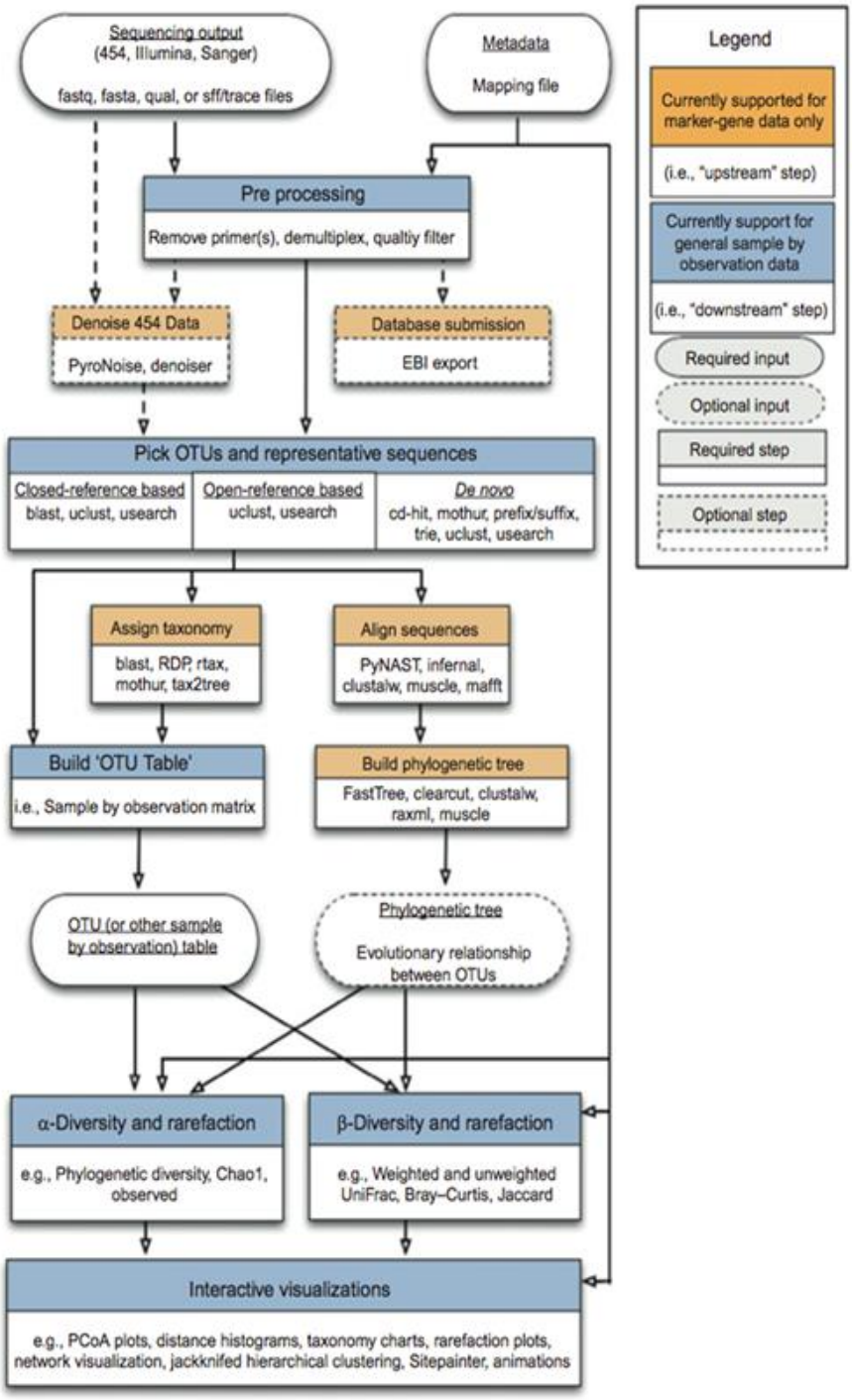
The preprocessing step defines parameters that are used to include or exclude sequences from the data set, and includes demultiplexing, primer removal, and quality filtering of sequences (Figure 2.3). The demultiplexing step assigns samples based on their unique nucleotide barcode, to their mapping file. The main information in this mapping file consists of unique identifiers for each sample, the barcode used for each sample, the primer sequence used, and a description for each sample (eg time point, diet treatment, etc.). During demultiplexing, QIIME removes the barcodes and primer sequences. Quality filtering defines which sequences are good enough to be included in analysis and is based on cut-off for sequence length, end-trimming, or minimum quality score. In addition, for the 454 platform, sequences must be preprocessed using a de-noising step. For this, the QIIME Denoiser is used, which reduces the impact of homopolymer runs (runs of the same base) in data sets (Reeder and Knight, 2010). Lastly, during the PCR amplification process, some of the amplified sequences can be produced from multiple parent sequences, generating sequences known as chimeras. These sequences are technical artifacts and need to be removed from data sets using programs such as ChimeraSlayer (Haas et al., 2011) and USERCH6.1 (Edgar et al., 2011).

After preprocessing, sequences are analyzed to define the number of operational taxonomic units (OTUs). QIIME supports three approaches for OTU selection, including de novo OTU selection, closed-reference OTU selection, and open-reference OTU selection. The de novo OTU selection compares sequences that are only clustered against one another. The closed-reference OTU selection matches sequences to an existing database (eg. SILVA) of reference sequences. If a sequence fails to match the database, it is discarded. The open-

reference OTU selection process tries to match the sequences against an existing database, but if the sequence does not match the database it is retained and clustered de novo. Sample sequences that do not match those in the reference database are then classified as unknowns.

The next step in the bioinformatics analysis is to perform a template sequence alignment. The sequences must be aligned to infer a phylogenetic tree, which is used for diversity analyses. Currently, QIIME supports the following methods for performing sequence alignment: PyNAST (Caporaso et al., 2010), Infernal (Nawrocki et al., 2009), and muscle (Edgar, 2004). However, the recommended, and default method in QIIME is PyNAST. A phylogenetic tree is generated by a sequence alignment step, which defines the relationships among sequences. The microbial profiles obtained after this step contain various hierarchical levels of taxonomy classification, and their positions in the taxonomy tree are used to assess the degree of diversity within a microbial community (Hang et al., 2014).

The main output from the QIIME pipeline is the OTU table, which describes the microbial OTUs and their abundances in each of the samples. In addition, , alpha (within a sample characteristics) and beta diversity (the diversity between samples) measurements to look for community-level differences, and also visualization by PCoA plots, distance histograms, taxonomy summarization, OUT heatmaps, rarefaction plots, OUT network visualization, and jackknifed hierarchical clustering are provided.



By Knight. Using QIIME to analyze 16S rRNA gene sequences from Microbial Communities. (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3249058/)

Figure 2.3. Summary of workflow of qiime.

2.12 Application of NGS in ruminants

There are more than 3.5 billion domesticated ruminants including cattle, sheep. Goats, and buffalo (http://faostat.fao.org/) which are important sources of meat, milk, and other products to human. Since NGS techniques have been introduced, ruminant studies have used the technology in an effort to improve the overall understanding of the structure and function of microbial communities in the rumen (Sirohi et al., 2012). These studies have demonstrated that feed digestibility and host health are dependent upon the rumen microbial community (McCann, et al., 2014). Rumen bacteria have been classified into three groups according to the rumen environment, including; (1) free-living bacteria associated with the rumen liquid phase, (2) bacteria associated with feed particles, and (3) bacteria attached with rumen epithelium. The liquid fraction and solid fraction samples have been frequently analyzed because these two fractions are largely involved in the digestibility of feed particles in the rumen (Firkins, 2010). This work has shown that he composition of liquid and solid associated rumen bacterial communities differ. Using pyrosequencing, McCann, (2013) showed that beef steers on a low-quality forage diet had higher relative abundances of the *Prevotella* and *Treponema* in the solid as compared to the liquid fraction of rumen contents. In contrast, *Paludibacter* and *Succiniclasticum* were greater in the liquid than the solid fraction. In an analysis of elk rumen samples by 454 pryosequencing,, *Bacteroidetes* were more predominant in the liquid phase whereas *Firmicutes* was the most prevalent phyla in the solid fraction (Gruninger et al., 2014). There were also significant differences in the abundance of OTUs classified as *Fibrobacteres* and *Spirochaetes* in the solid fraction than in the liquid fraction of these elk samples (Gruninger et al., 2014). These studies support the theory that bacterial communities differ between the solid and liquid phases of rumen contents.

Previous studies have also shown that the rumen microbial communities are affected by diet, host, and geographic range, but that diet is the most important factors affecting the composition of rumen microbiota (Thoetkiattikul et al., 2013; Petri et al., 2013a,b). When dairy cows were fed a high fibre diet, the fibrolytic and cellulolytic bacteria, *Lachnospiraceae*, *Ruminococcaceae*, and *Fibrobacteraceae* were found in highest abundance (Thoetkiattikul et al., 2013). However, when cows were adapted to a o high starch diett, polysaccharide-degrading *Prevotellaceae* and *Flavobacteriaceae* bacteria were most abundant in the rumen (Thoetkiattikul et al., 2013). Similarly, Pitta et al., (2010) showed that switching cowss from bermudagrass hay to a wheat forage diet affected both the composition and diversity of the rumen bacterial population when fed wheat forage, *Prevotella* was dominant and *Rikenella* decreased compared to when cattle were fed bermudagrass hay. Moreover, *Tannerella* and *Succiniclasticum* increased with the wheat forage diet. In addition, starch and oil content in the diet has been shown to affect rumen bacteria in cattle using NGS analysis (Zened et al., 2013). A high abundance of *Prevotellaceae* and low abundance of *Ruminococcaceae* and *Rikenellaceae* were found when cattle were fed a high-starch plus 5% sunflower oil diet compared to a low starch diet that lacked sunflower oil (Zened et al., 2013).

2.13 Conclusions

Barley is the primary feed grain for ruminants in western Canada because it is well adapted for growth in this region, has more protein than other cereals commonly used in ruminant diets and is highly digestible in the rumen. However, the composition of barley can be affected by genetic background and the conditions under which it is grown. Variation in nutritional composition can result in different digestibilities in the rumen through alteration of ruminal microorganisms, which have highly specific mechanisms to colonize and digest feeds. The digestibility of barley grain can be improved by processing, which alter the physical structure and allow greaten microbial access to the starch endosperm for digestion. Previous studies characterizing the ruminal microorganisms involved in starch digestion were mostly based on traditional culture techniques for identification and enumeration of microbial populations. However, culture-based techniques can only study the cultivable microbes that will grow on defined media. Recent advances in molecular methods, such as NGS technologies, are able to produce vast amounts of sequence data. Combined with proper bioinformatics pipelines, NGS data have the potential to provide novel information on the non-culturable bacterial species that contribute to microbial diversity of the rumen and have important roles in the digestion of barley.

2.14 Hypothesis

It is hypothesized that the population structure of rumen microbial biofilms will differ among barley varieties that differ in their rate and extent of ruminal digestibility and in their chemical composition.

2.15 Objectives

1) To estimate the temporal *in situ* rumen digestion of four barley grain varieties.

2) To evaluate the temporal formations of bacterial biofilms involved in the digestion of differential varieties of barley grain.

3.0 COMPARISON OF DIGESTION AND PARTICLE-ASSOCIATED BACTERIA (PAB) AFTER IN SITU INCUBATION OF DIFFERENT BARLEY VARIETIES IN THE RUMEN OF CATTLE

3.1 Introduction

Barley ranked as the fourth major cereal crop in the world is consumed by humans and livestock (Nikkhah, 2012) and is the primary grain fed to ruminants in western Canada (Legesse et al. 2015). On average, barley grain is composed of about 60% starch, 20% fibre and 12% protein on a DM basis (Åman and Newman, 1986; Oscarsson et al., 1996). However, the chemical composition, nutritive value and bioavailability of starch can vary among barley varieties due to both genetic and environmental factors (Nilan and Ullrich, 1993; Nikkhah, 2012).

Barley grain can be classified as two or six row, hulled or hulless, and on the basis of starch type as normal, waxy (high amylopectin up to 100%), and high-amylose (amylose up to 70%). Generally, two-row barley has higher protein and starch and lower β-glucan and fibre concentrations than six-row barley (Nikkhah, 2012; Narasimhalu et al., 1995; Kong et al., 1995). Hulless barley typically has a higher total protein, starch, and digestible energy content, as the absence of the hull lowers the amount of fibre in the kernel (Nikkhah, 2012).

Variations in the chemical composition and structure of barley grain can affect its digestibility in the rumen. Cleary et al. (2011) showed that two varieties of barley grain differed in their ruminal DM digestion kinetics. The soluble DM fraction ranged from 22.9-32.7%, the slowly degradable DM fraction ranged from 46.1-65.6%, and the undegradable DM fraction from 3.8-29.9%. The rates of digestion ranged from 12.7-16.5 $\%/h^{-1}$ and the *in situ* effective

degradability from 52.7-75.7%. Barley degradation in the rumen is dependent on the ability of rumen microbes to colonize and form biofilms on the surface of the grain (McAllister et al., 1994; Beauchemin et al., 2001). Formation of biofilms can be promoted by processing barley grain as this procedure exposes the more digestible components of the endosperm, enhancing microbial attachment and the colonization of starch granules (McAllister et al., 1994). However, even when processed using the same techniques, ruminal digestion can differ among barley varieties. Little is known about how differences in barley grain composition affect the types of rumen bacteria that form biofilms on the surface of barley grain.

Previous studies of the microbiota involved in the ruminal digestion of barley grain have primarily relied on culture-based methods and were therefore limited to only those bacteria that could be grown in the laboratory. Recent developments in molecular biology has led to a proliferation in the use of next generation sequencing (NGS) to characterize the ecology of microbial populations in a variety of ecological habitats, including the rumen (Petri et al., 2013a). NGS is a culture-free method that enables analysis of the entire bacterial population based on sequencing of the16S rRNA gene. It enables the characterization of the phylogeny and taxonomy of bacteria including those that are associated with complex biofilms. The purpose of this study was to evaluate the bacteria associated with biofilms on the surface of different varieties of barley using 16S rRNA gene sequencing.

We hypothesized that the population structure and density of rumen microbial biofilms differ among barley varieties as a result of difference in their chemical composition. Corn was included in the study as a positive control as it is well known that the chemical composition of corn is different from barley (Peter and Herbert, 2013).

3.2 Material and Methods

3.2.1 Animal and diets

Three rumen-cannulated beef heifers (BW: 308kg ± 32 SD) were individually housed for the duration of the experiment and had free access to clean drinking water. The heifers were fed *ad libitum* twice daily at 08:00 and 16:00 h. The study was reviewed and approved by the Lethbridge Research Centre Animal Care Committee and conducted according to the guidelines of the Canadian Council on Animal Care (1997). In the first experiment, heifers were adapted to a diet (DM basis) consisting of 60% barley silage, 37% steam rolled barley and 3% of a standard feedlot supplement for a period of 21 d (low grain diet; Table 3.1). *In situ* incubations were conducted and then heifers were adapted to a high concentrate diet over 14 d. The final diet consisted of 37% barley silage, 60% steam rolled barley and 3% supplement (high grain diet; Table 3.1). Heifers were fed the final diet for an additional 14 d prior to *in situ* incubations. Barley varieties were obtained from the Crop Development Centre in Saskatoon, Saskatchewan, and feed corn was obtained from a feedmill in southern Alberta.

Table 3.1. Diet composition

Composition	Low concentrate	High concentrate
Ingredients (% of dry matter)		
Barley silage	60	37
Barley grain	37	60
Supplement [a]	3	3
Composition (% of dry matter)		
Crude protein	12.0	12.1
Acid detergent fiber	24.9	11.8
Neutral detergent fiber	36.2	19.7
Starch	16.7	35.2

[a] The supplement contained canola meal (10%), urea (2%), calcium carb (25%), sodium chloride (3%), ground barley grain (56.5%), Molasses (2.5%) and feed lot premix (1%). The premix contained calcium carbonate (34.8%), zin sulfate (28.4%), manganous sulfate (14.6%), copper sulfate (10.3%), ethylene diaminediiodic acid (0.2%; as an 80% preparation), selenium (5%), cobalt sulfate (0.1%), vitamin A (1000 000 IU g-1; 1.7%), vitamin D (500 000 IU g-1; 0.2%) and vitamin E (4.7%).

3.2.2 Incubation of barley varieties and corn in the rumen

Prior to placement in nylon bags, barley varieties (McGwire, Xena, Fibar, and Hilose) and corn samples were ground using a Wiley mill (Arthur H. Thomas Co.) fitted with a 6 mm screen. One set of bags (3 heifers x 3 bags/time point/treatment; n=9) was used to estimate dry matter (DM), starch and crude protein (CP) disappearance. Samples of ground grain with a 6mm screen (3 g) were hot weighed into 50μm pore size (#R510) Dacron nylon bags (5 cm x 10 cm; Ankom, Fairport NY, USA) and placed into a larger mesh bag. A second set of bags (2 heifers x 3 bags/time point/ treatment; n=6) containing 5 g of ground grain were incubated and used for DNA extraction. A third set of bags (2 heifers x 2 bags/time point/treatment; n=4) containing ground grain (5 g) were incubated and examined using scanning electron microscopy (SEM). The same two heifers were used for DNA and SEM bags. Bags to estimate nutrient digestion were incubated for 0, 2, 4, 12, 24 and 48 h and for 2, 4, and 12 h for DNA extraction and SEM. Bags used to estimate nutrient disappearance were retrieved from the rumen and thoroughly rinsed with cold tap water until the water was clear. Bags were then washed in a washing machine for 2 min without the spin cycle, prior to being dried at 55 °C for 48 h. For DNA extraction, samples were rinsed with phosphate buffer three times to remove non-adherent bacteria and bags along with their contents were flash-frozen in liquid nitrogen. For SEM, a few random particles from the bags were placed into a prefix solution containing 0.5% glutaraldehyde, 0.15% ruthenium red in 0.2M phosphate buffer saline at a pH of 7.2. Zero hour bags were not placed in the rumen, but were washed in the same manner as the other bags.

3.2.3 DNA extraction of bacterial biofilms

Frozen samples (2 heifers x 3 bags; n = 6) were freeze-dried and ball-ground using a Tissue Lyzer (Qiagen, Germany). DNA was extracted using a Qiagen QIAamp DNA stool mini kit with minor modifications (Qiagen, Hilden, Germany). Briefly, 300 mg of ground substrate were placed into a 2 ml micro centrifuge tube along with 1.4 ml of ASL buffer (cell lysis) and the mixture was vortexed for 1 min. Glass beads (200 mg with 0.5 mm diameter and 300 mg with 1.0 mm diameter) were added to each tube and the samples were processed in a bead-beating homogenizer (B. Braun, Melsungen AG, Germany) for 3 min at maximum amplitude. The tubes were then centrifuged (13,000 x g, 5 min) and subsequently extracted following the QIAamp DNA stool mini kit instructions. After extraction, DNA was quantified using a NanoDrop 3300 (Thermo, Waltham, MA, USA).

3.2.4 Pyrosequencing of the 16S rRNA gene

Extracted DNA was analyzed using bacterial tag-encoded FLX 16s rRNA amplicon pyrosequencings (bTEFAP). The 16S rRNA gene universal Bacterial primers 27F-519R (27F 5′-AGRGTTTGATCMTGGCTCAG, 519R 5′-GTNTTACNGCGGCKGCTG), were used to amplify the hypervariable regions V1 to V3 of the 16S rRNA gene as described by Dowd *et al.* (2008). All DNA samples were adjusted to100 ng/µl and an 1 µl aliquot (100 ng) of each sample of extracted DNA was used in a 50 µl PCR reaction. Reactions were performed using a HotStarTaq plus Master Mix Kit (Qiagen, Valencia, CA) with the following conditions: 94^{o}C for 3 min, followed by 28 cycles of 94^{o}C for 30 sec; 53^{o}C for 40 sec and 72^{o}C for 1 min; followed by a final elongation step at 72^{o}C for 5 min. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt

Ampure beads (Agencourt Bioscience Corporation, MA, USA). Prepared libraries were sequenced utilizing Roche 454 FLX titanium (454 Life Sciences, a Roche company, Branford, CT, USA) technology with reagents and execution of the procedure as per manufacturer's guidelines.

3.2.5 Sequencing analysis by QIIME 1.9.1

16S rRNA gene sequences were processed and analyzed with the QIIME software package v. 1.9.1 (Caporaso et al., 2010). All samples were demultiplexed and quality filtered based on the characteristics of each sequence (such as the removal of primers and barcodes), low quality or ambiguous reads and sequences with an average Phred score of < 25 were removed. Chimeric sequences were removed using the UCHIME algorithm implemented in USEARCH (version 6.1544). The remaining high quality 16S rRNA gene sequences were clustered into OTUs (operational taxonomic units) at 97% similarity using the de novo reference OTU picking method and USEARCH (version 6.1544). Taxonomy was assigned using the UCLUST consensus taxonomy assigner (Edgar, 2010). PyNAST (Caporaso et al., 2010) was used to align the representative sequences for each OTU and a phylogenetic tree was created using FastTree (Price et al., 2010).

3.2.6 Chemical analysis of barley grain varieties and corn

Briefly, lipids were determined using the Goldfisch extraction apparatus (Labconco Corporation, Kansas, MO, USA) with petroleum ether as the solvent (AOAC 2010). Total nitrogen (TN) was measured by combustion analysis using a Leco® Carbon/Nitrogen analyzer (TruSpec® CN, Leco Corporation, St. Joseph, MI, USA), with crude protein calculated as TN x 6.2. Soluble dietary fibre (SDF) and insoluble dietary fibre (IDF) were quantified using the

enzymatic gravimentric procedures of AOAC Methods 993.19 and 991.42, respectively (AOAC 1997) using Megazyme's total dietary fiber assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland). Total dietary fibre (TDF) was calculated as SDF plus IDF. Contents of starch and β-glucan were estimated according to the total starch (AA/AMG) and the mixed-linkage β-glucan assays, respectively (Megazyme International Ireland Ltd). Amylose content of starch was determined using the iodine blue method as defined by the Corn Refiners Association (1997). Starch content of the grain residues remaining after ruminal incubation was determined by hydrolyzing starch to glucose polymers using amyloglucosidase (Megazyme International Ltd,Wicklow, Ireland) plus 1,4-α-d-glucan glucano-hydrolase (Brenntag Canada Inc., Toronto, ON) as described by Herrera-Saldana et al. (1990). For measurement of starch, samples were ground using a ball mill (Mixer Mill MM 2000, Retsch, Germany) to a fine powder. Samples (0.1g) were weighed into 50-ml test tubes and 25 ml of 0.1 N Na-acetate buffer (pH 5.0, Ca2+ 70 ppm) was added. Amylase (200μl; Termamyl, Novo Nordisk, Bagsvaerd, Denmark) was added and tubes were vortexed immediately and subsequently at 15-min intervals. For starch determination, tubes were incubated in a water bath (98°C) for 1 h to gelatinize and hydrolyze starch. Activated carbon (approximately 0.04 g; Darco_ G-60 Activated Carbon, Fisher Scientific Co., No D-127) was added to the tubes, which were then placed for 15 min into at 60°C water. Amyloglucosidase (500 μL, Boehringer Mannheim, Laval, QC, Canada, no. 208-469) was added, tubes were vortexed immediately and twice subsequently at hourly intervals, while being held in a 60°C water bath until the next morning. Samples were centrifuged (695 × *g* for 10 min), 500 μl of supernatant was diluted with 9.5 ml of distilled water, and 50 μl was transferred to a micro plate. Glucose trinder reagent was added (150 μl, Sigma Chemical Co. No.

315-100), and released glucose was determined colorimetrically at 490 nm using a microtiter plate reader.

3.2.7 Scanning electron microscopy (SEM)

Residues of barley grain and corn were fixed in a pre-fix solution (0.5% glutaraldehyde and 0.15% ruthenium red in 0.2M phosphate buffer, pH 7.2). After 2 h, feed particles were removed from the pre-fix solution, and a fix solution of 5% glutaraldehyde and 0.05% ruthenium red in 0.2M phosphate buffer (pH 7.2) was added so as to cover the sample. The fix solution was removed after 2 h and a wash solution (0.2M phosphate buffer and 0.05% ruthenium red, pH 7.2) was added and incubated for 20 min. The wash step was repeated two more times, the final wash solution was removed and a post-fix solution was added (2% OsO4 and 0.05% ruthenium red in 0.2M phosphate buffer, pH 7.2). The sample was gently shaken for 2 h and washed 5 times with wash solution at 10 min intervals. Samples were then dehydrated using a graduate ethanol series containing 10%, 20%, 30% 50%, 70%, 95%, and 100%, ethanol with samples allowed to stand for 15 min at each increment. The specimens were mounted on aluminium stubs with silver paste, coated with gold and observed using a Hitachi S-570 scanning electron microscope (Hitachi High Technologies, Tokyo, Japan) at an accelerating voltage of 19 kV and were photographed with images digitally captured using Quartz PCI software (Quartz Imaging Corporation, Vancouver, British Columbia, Canada). Scanning electron microscopy data were evaluated based on magnification rates indicated in the images.

3.2.8 *In situ* digestibility of four barley varieties and corn

In situ DM disappearance of each grain type was determined gravimetrically as the difference between initial and remaining weight of DM after ruminal incubation at each time point. Kinetic parameters of DM, starch, and protein were calculated using the equation (McDonald, 1981):

$$D = a + b\,(1-e^{-c(t-lag)}) \quad \text{for } t > lag$$

Where D = ruminal disappearance at time t, a = the rapidly degradable fraction, b = the slowly degradable fraction, c = the rate at which b was degraded (/h), t = time of incubation (h), and lag = lag time (h). The parameters a, b, c and lag were estimated using NLIN procedure of SAS (SAS Institute Inc., 2012). Effective degradability (ED) of dry matter, starch, and protein were estimated using the equation of Ørskov and McDonald (1979), ED = a + bc/(c+k), where k was the ruminal passage rate of 0.06/h. Digestibility was represented as a percentage of substrate remaining at each time point compared to the initial substrate weight, on a dry matter basis.

All data for each grain type associated with each diet were analysed as a completely randomized design using PROC MIXED procedure of SAS (2012). For the *in situ* study, the mixed model included the fixed effects of grain type and the random effects of heifer and sample. Replicates within heifer were averaged prior to statistical analysis. Results from the two diet types were analysed separately. Effects of fixed factors were tested using LSMEANS with the PDIFF in SAS (2012) and significance was declared at $P<0.05$. Trends were discussed at $0.05 < P < 0.10$ unless otherwise stated.

3.2.9 Rumen particle-associated bacteria sequencing analysis

Rarefication plots are shown in Supplementary Figure 1. To account for uneven sequencing depth across samples, each sample was randomly subsampled to 6,292 and 2,710 in the low and high grain experiments, respectively. For sequencing data, bacterial populations were calculated as relative abundances (number of OTU reads / rarefied sample sizes). Bacterial diversity within each sample (alpha-diversity) was calculated within QIIME using the Chao1estimator (Chao 1984), observed species (observed OTUs), Shannon's diversity index (Shannon 1948), and PD whole tree (phylogenetic diversity whole tree) (Faith, 1992) metrics. The bacterial community structure (beta-diversity) within biofilms from all four barley varieties and corn was evaluated using the unweighted and weighted UniFrac distances (Lozupone and Knight, 2005) and visualized as principal coordinate analysis (PCoA) plots using Emperor (Vazquez-Baeza et al., 2013). ANOSIM (analysis of similarities) with 999 permutations was used to compare the unweighted UniFrac distances.

Linear discriminant analysis effect size (LefSe) was used to determine which taxa group differed according to incubation time of corn and barley in heifers fed low and high grain diet. LefSe includes the Kruskal-Wallis test to identify significantly different ($P < 0.05$) taxa among groups of samples followed by linear discriminant analysis which estimates the effect size of each of these differences (Segata et al., 2011).

3.3 Results

3.3.1 Chemical composition of barley varieties and corn

For this study, four barley grain varieties (CDC Fibar, CDC Hilose, CDC McGwire and Xena) were selected based on differences in their compositions, and corn was used as a standard

of comparison for *in situ* digestibility in the rumen (Table 3.2). McGwire and Xena were normal starch varieties (25% amylose and 75% amylopectin); CDC Fibar, was a waxy type and had a high β-glucan, protein, soluble dietary fibre (SDF), total dietary fibre (TDF) content but low starch content. CDC Hilose was a high-amylose type with 63 % of the starch composed of amylose and also contained the highest fat content. Corn, was higher in starch, fat, and insoluble dietary fibre (IDF) than barley, but lower in β-glucan, protein, SDF and TDF.

Table 3.2. Chemical composition of four different barley grain varieties and corn (values represent mean percentage with standard deviation from triplicate measurements (% of dry matter)

						Dietary fibre		
Grain types	BG[a]	Starch	Amylose	Protein	Fat	SDF[b]	IDF[c]	TDF[d]
Fibar	10.1±0.12	47.8±0.08	0.2±0.01	15.6±0.06	2.6±0.04	7.9±0.17	11.0±0.24	18.9±0.21
McGwire	4.6±0.07	52.5±0.18	11.5±0.88	12.7±0.01	2.0±0.05	5.6±0.38	8.6±0.21	14.2±0.58
Hilose	6.6±0.14	49.0±0.03	18.0±0.25	12.6±0.08	3.0±0.14	7.4±0.31	9.5±0.20	16.9±0.51
Xena	4.1±0.16	56.1±0.15	11.2±0.28	11.4±0.05	1.6±0.05	5.1±0.41	10.4±0.54	15.5±0.15
Corn	0.31±0.00	62.2±0.44	17.1±0.74	7.9±0.03	3.95±0.1	1.14±0.10	12.2±0.25	13.3±0.14

[a]BG, [b]SDF, [c]IDF and [d]TDF represent β-glucan, soluble dietary fiber, insoluble dietary fiber and total dietary fiber, respectively.

3.3.2 Comparisons of digestibility after *in situ* incubation of different barley varieties in the rumen when heifers were fed low and high grain diets

With the low grain diet, the rapid (A) and slow degradable (B) fractions, degradation rate (Kd), the potential degradable fraction (A+B) and effective degradability (ED) of dry matter differed ($P<0.01$) among grain types (Table 3). For DM, the A fraction was the highest ($P<0.01$) for corn (18.2%; Table 3; Figure 1A) and the lowest for Fibar (11.3%). The fraction B of DM was the highest ($P<0.01$) for Hilose (81.6%) and lowest for Xena (73.3%). Degradation rate (Kd) of DM was the fastest ($P<0.01$) for Xena (15%/h) and slowest for Corn 4.0%/h). The potential degradability fraction (A+B) of DM ranked as Hilose>Corn>McGwire>Fibar>Xena. The highest values ($P<0.01$) of ED were observed with McGwire and the lowest with Corn. For starch, the rapidly degradable fraction (A) was highest for Fibar ($P<0.01$; Table 3; Figure 1C) 22.5%. Hilose was the lowest fraction (A) and highest the slowly degradable fraction (B) at 7.0% and 91.4%, respectively. The degradation rate (Kd) of starch was fastest for Xena (14%/h) and Fibar (12%/h). The effective degradability (ED) of starch was highest ($P<0.01$) for Fibar (70.8%) and Xena (70.7%), and lowest ($P<0.01$) for Corn (43.7%). For protein, the rapidly degradable fraction (A) was not different among grain types (Table 3; Figure 1E). The slowly degradable fraction (B) was highest ($P<0.01$) for Corn (99.1%) and lowest for McGwire (83.9%). However, the degradation rate (Kd) of protein did not differ among grain types. The effective degradability (ED) of protein, Corn (25.4%) was lower than barley grains.

When heifers were fed the high grain diet, the rapidly degradable DM fraction (A) was highest ($P<0.01$) for Corn (20.2%; Table 3; Figure 1B) and lowest for Fibar (11.8%). The degradation rate (Kd) of DM was faster ($P<0.01$) for Xena (14%/h) and lowest for Corn (4.0%/h). The potential degradable DM fraction (A+B) ($P<0.01$) ranked as Corn=Fibar=Hilose=

McGwire>Xena, but Xena (60.1%) and McGwire (59.1%) had a higher effective degradability (ED) (P<0.01) than Hilose and Corn. The rapidly degradable fraction (A) of starch was highest for Fibar (21.1%; P<0.01; Table 3; Figure 1D). Xena had the highest degradation rate (Kd) and effective degradability (ED) of starch at 11%/h and 66.3%, respectively. The degradable fraction A, B, and Kd of protein had no significant effect among grain types. The effective degradability (ED) of protein, Fibar (55.0%) showed the highest ED than other barley grains (Table 3; Figure 1F).

Table 3.3. *In situ* degradability estimates of a corn and four barley varieties when heifers fed either a low or high grain (n=9 per time point per treatment).

Low grain diet	Corn	Fibar	Hilose	McGwire	Xena	SEM
Dry Matter						
A (%)	18.16^{a}	11.33^{c}	13.19^{b}	12.47^{bc}	12.26^{bc}	0.35
B (%)	75.66^{bc}	79.60^{ab}	81.63^{a}	78.22^{abc}	73.29^{c}	1.34
Kd (/h)	0.04^{d}	0.12^{b}	0.07^{c}	0.14^{a}	0.15^{a}	0.00
A+B (%)	93.82^{a}	91.00^{ab}	94.81^{a}	90.69^{ab}	85.55^{b}	1.37
ED(6%/h)	44.99^{d}	63.86^{b}	55.56^{c}	67.49^{a}	64.66^{b}	0.45
Starch						
A(%)	12.50^{b}	22.54 ^{a}	7.00^{b}	12.04^{b}	10.04^{b}	1.09
B(%)	85.31^{ab}	73.42^{b}	91.43^{a}	84.87^{ab}	87.52^{ab}	1.25
Kd(/h)	0.03^{d}	0.12^{a}	0.07^{c}	0.10^{b}	0.14^{a}	0.00
ED(6%/h)	43.70^{c}	70.83^{a}	55.48^{b}	65.58^{a}	70.69^{a}	0.68
Protein						
A(%)	0.86	0.32	2.77	2.25	4.42	1.14
B(%)	99.14^{a}	88.54^{ab}	90.26^{ab}	83.86^{b}	87.89^{ab}	1.09
Kd(/h)	0.02	0.10	0.06	0.10	0.09	0.00
ED(6%/h)	25.39^{b}	56.51^{a}	47.56^{a}	54.09^{a}	54.24^{a}	1.31

High grain diet	Corn	Fibar	Hilose	McGwire	Xena	SEM
Dry Matter						
A(%)	20.17^{a}	11.82^{d}	14.74^{b}	13.29^{c}	13.29^{c}	0.35
B(%)	68.82	73.08	70.19	72.24	67.37	1.59
Kd(/h)	0.04^{d}	0.10^{bc}	0.08^{c}	0.13^{ab}	0.14^{a}	0.01
A+B(%)	88.99^{a}	84.90^{ab}	84.92^{ab}	85.53^{ab}	80.66^{b}	1.59
ED(6%/h)	44.64^{c}	56.61^{ab}	52.65^{b}	59.05^{a}	60.12^{a}	1.45
Starch						
A(%)	12.22^{b}	21.14 ^{a}	10.08^{b}	11.93^{b}	13.34^{b}	1.30
B(%)	74.47^{ab}	69.72^{b}	80.27^{a}	77.05^{ab}	80.98^{a}	1.06
Kd(/h)	0.05^{b}	0.09^{a}	0.05^{b}	0.09^{a}	0.11^{a}	0.01
ED(6%/h)	43.97^{c}	63.85^{a}	46.96^{c}	58.77^{b}	66.25^{a}	1.04
Protein						
A(%)	6.53	0.86	5.81	5.59	6.28	1.48
B(%)	72.14	85.06	76.03	77.90	82.90	0.45
Kd(/h)	0.03	0.10	0.08	0.09	0.08	0.00
ED(6%/h)	31.99^{b}	54.96^{a}	48.07^{ab}	51.61^{ab}	53.23^{ab}	2.05

^{A,b,ab,c,d} Means in the same row with different superscripts differ (P<0.01). A = the fast degradability fraction; B = the slowly (insoluble) degradability fraction; Kd = degradation rate; A+B = the potential degradability fraction; ED=effective degradability of dry matter, starch and protein; 1/Effective degradability in the rumen (assuming rate of passage of $0.06/h^{-1}$)

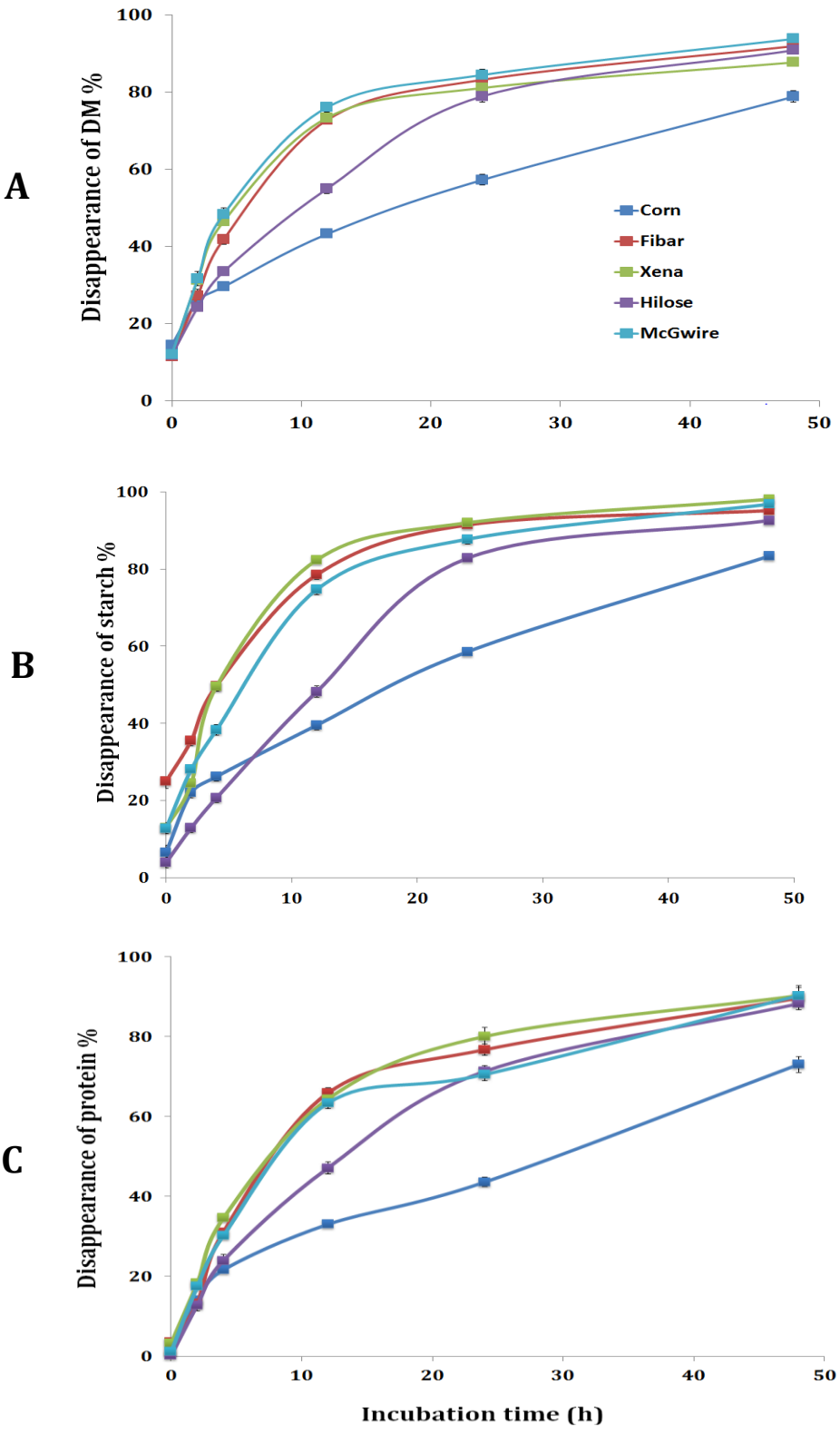


Figure 3.1. *In situ* percent DM, starch, and protein disappearance at six different ruminal incubation times when heifers were fed low grain diet (n=9 per time point per treatment).

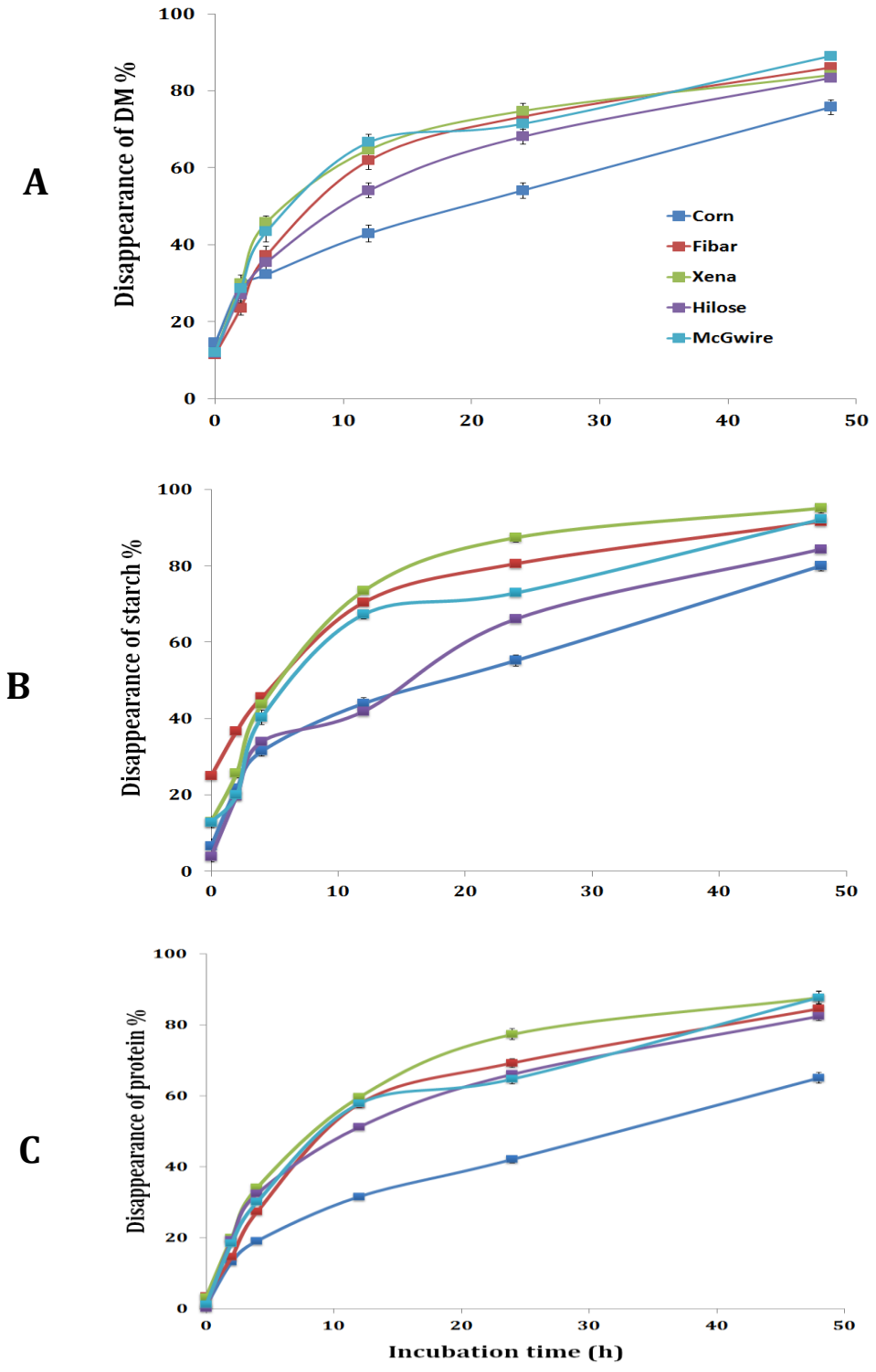


Figure 3.2. *In situ* percent DM, starch, and protein disappearance at sixdifferent ruminal incubation times when heifers were fed high grain diet (n=9 per time point per treatment).

3.3.3 Differences in particle-associated bacteria (PAB) between corn and the four barley varieties when heifers were fed a low grain diet

A total of 604,714 raw sequences were generated from the corn and four barley varieties collected from heifers fed the low grain diet. After trimming, quality filtering and chimera removal, a total of 464,120 sequences remained, with an average length of 421 bp. With a cut-off of 97% sequence similarity, 945 bacterial OTUs were obtained. The 945 OTUs were classified into 10 phyla, 29 families, 51 genera and 1 unclassified group. Species diversity, richness and evenness were calculated using Chao1 (species richness), observed species (observed OTUs), PD whole tree (phylogenetic diversity whole tree)**,** and Shannon's diversity index (Figure 3.3) across the three time points (2, 4 and 12h). We found no difference in observed species or Shannon diversity between corn and barley varieties. However, there was a tendency for Choa1 ($P = 0.06$) and PD ($P = 0.08$) to differ between corn and barley varieties. Based on a Jackknife similarity plot at 97% identity, estimated hierarchical clustering of the bacterial microbiome associated with corn and barley varieties are shown in Figure 3.4. Based on this, the bacterial communities at 2 and 4 h incubation time were more similar than those after 12 h of incubation

The relationships between the bacterial community structure of the biofilm of corn and barley varieties were examined by using the Principal Coordinate Analysis (PCoA) based on the unweighted Unifrac distance matrices (Figure 3.5). The bacterial communities were not different among barley varieties (Figure 3.5A), but interestingly, the bacterial communities associated with corn and barley grains at 12 h of incubation tended to separate from 2 and 4 h of incubation (Figure 3.5B). However, after 2 and 4 h of incubation the bacterial communities of corn and barley grains did cluster separately between heifers (Figure 3.5C).

Although the bacterial communities did not differ among barley varieties, there were noticeable differences in the bacterial communities during the incubation period in the rumen (Figure 3.6). At 2 h, 15 genera were noticeable higher in the biofilm associated with barley grain as compared to after 4 and 12 h of incubation. After 4 h of incubation, *Sharpea, Coprococcus*, and *Ruminobacter* were higher in biofilms associated with barley grain than after 2 and 12 h. After 12 h of incubation, *Lactobacillus* and *Megasphaera* were more abundant in the biofilm than at 2 and 4 h. In addition, Figure 3.7 showed that the increase in lactic acid-utilizing bacteria (*Megasphaera*; Figure 3.7A and *Selenomonas*; Figure 3.7B) corresponded to an increase in lactic acid produced bacteria (*Lactocaillus*; Figure 3.7C). This highlights the relationship between lactic acid producers and utilizers, and production and use of lactate as a substrate in the rumen.

At the phylum level, 11 phyla were observed in heifers fed low grain diet (Figure 3.8). After 2 h of incubation, Firmicutes and Bacteroidetes were the predominant phyla, followed by Proteobactera, Unclassified and Fibrobacteres. After 4 h of incubation, Firmicutes and Bacteroidetes were the predominant phyla as well. However, after 12 h of incubation, Firmicutes was the most dominant phylum in all samples, with a relative abundance of 89.2% in corn, 95.0% in Fibar, 95.2% in Hilose, 94.5% in McGwire, and 91.68% in Xena.

Prevotella was the most dominant genus accounting for 16.5, 6.1, 10.8, 6.3, and 7.8% in corn, Fibar, Xena, Hilose, and McGwire after 2 h of incubation, respectively (Figure 3.9A). After 4 h of incubation, *Prevotella* (16.3%) predominanted, followed by unclassified *Succinivibrionaceae,* unclassified *Lachnospiraceae, and Sharpea* in corn. In contrast, *Sharpea* was the most abundant in Fibar, Hilose, McGwire, and Xena, representing 17.3, 16.8, 17.9, and 19.1% of the population, respectively (Figure 3.9B). After 12 h of incubation, *Lactobacillus* was the most abundant in both corn and barley (Figure 3.9C).

The comparison of corn and barley grain showed that 3 genera such as Unclassified *Succinivibrionaceae*, *Ruminobacter*, and Unclassified *Ruminococcaceae* significantly increased in corn than barley grain (Figure 3.10A). At this time, four genera (*Succiniclasticum*, Unclassified SR1, Unclassified *Paraprevotellaceae*, and Unclassified *Lachnospiraceae*) were higher ($P<0.05$) in corn than barley whereas *Lactobacillus* and *Sharpea* were higher in barley than corn (Figure 3.10B). Moreover, at 12 h incubation, unclassified *Lachnospiraceae*, *Prevotella*, unclassified *Clostridiales*, *Selenomonas*, unclassified *Succinivibrionaceae*, unclassified SR1, *YRC22*, unclassifed YS2, and *Anaerovibrio* were higher ($P<0.05$) in corn than in the barley varieties (Figure 3.10C)**.**

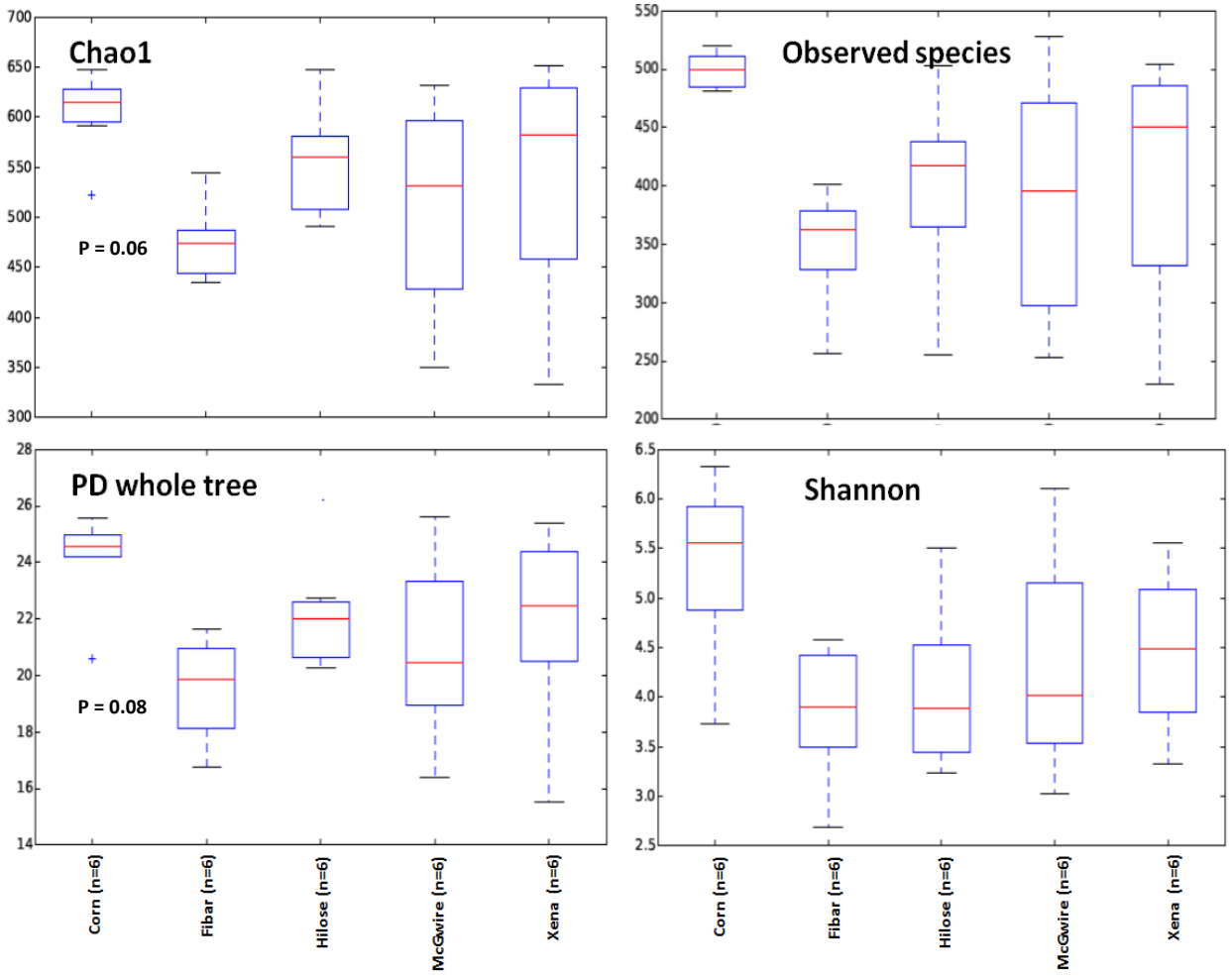


Figure 3.3. Alpha diversity measures of Chao1, Observed species, PD whole tree, and Shannon diversity index associated with biofilms on the surface of corn (n=6) and barley (n=6) per treatment in the rumen of heifers fed a low grain diet.

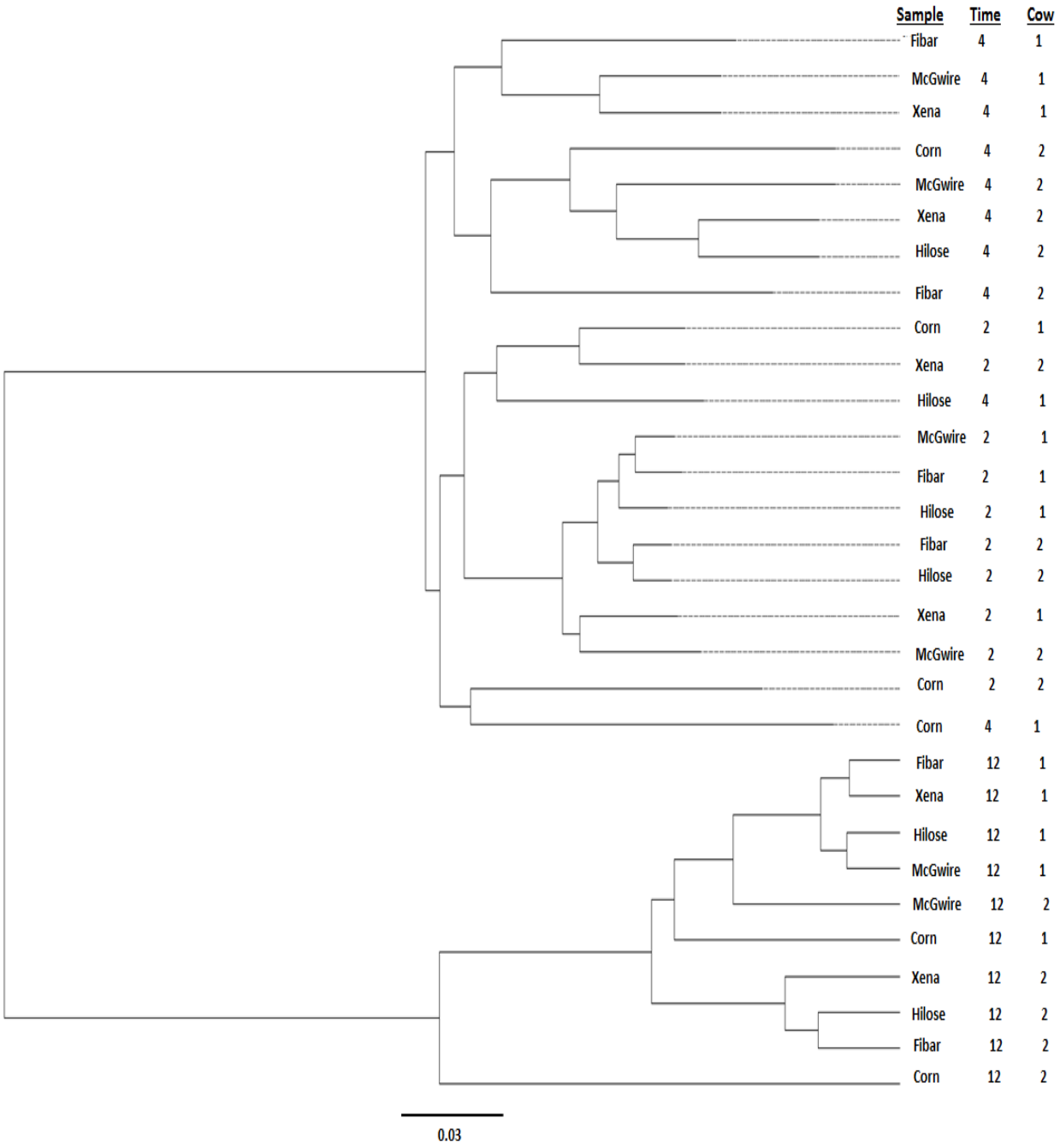


Figure 3.4. Dendrogram shows the relationships among the bacterial communities when heifers were fed low grain diet. The dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm and Bray-Curtis calculation for determining the distance between communities (n=6 per time point per treatment).

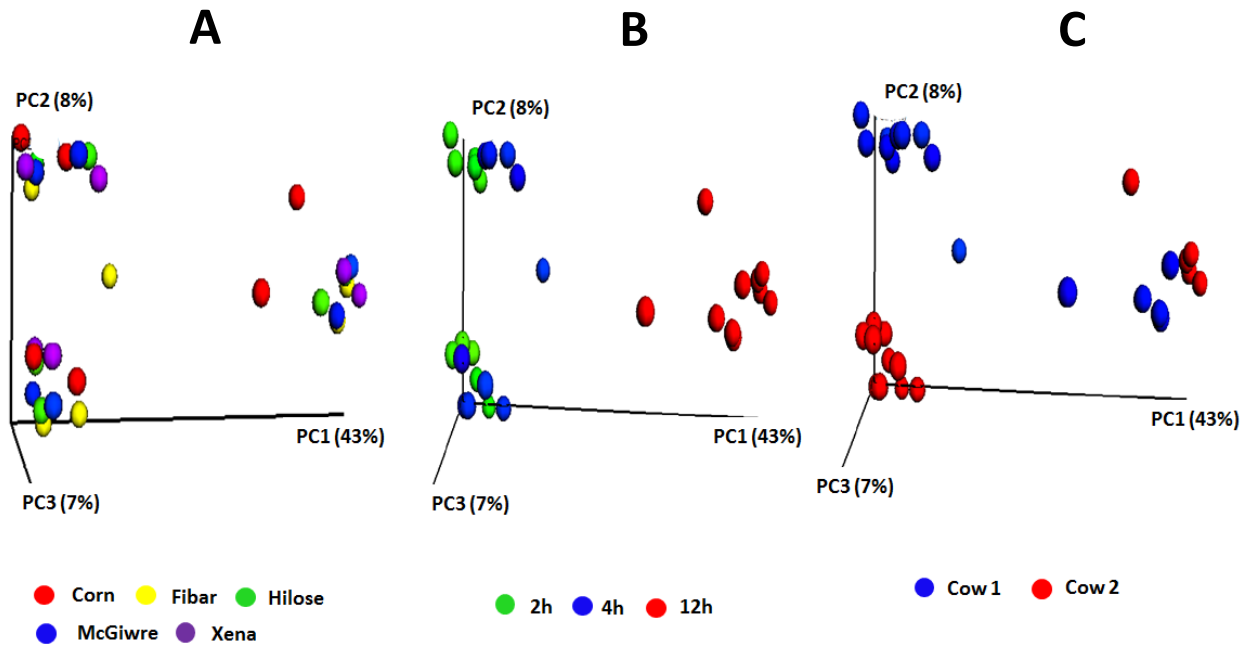


Figure 3.5. Principle coordinate analysis (PCoA) plots of the unweighted UniFrac distances for PAB of a corn and four barley varieties in heifers fed low grain diet A) a corn and four barley varieties, B) sampling time and C) sampling animal (n=6 per time point per treatment).

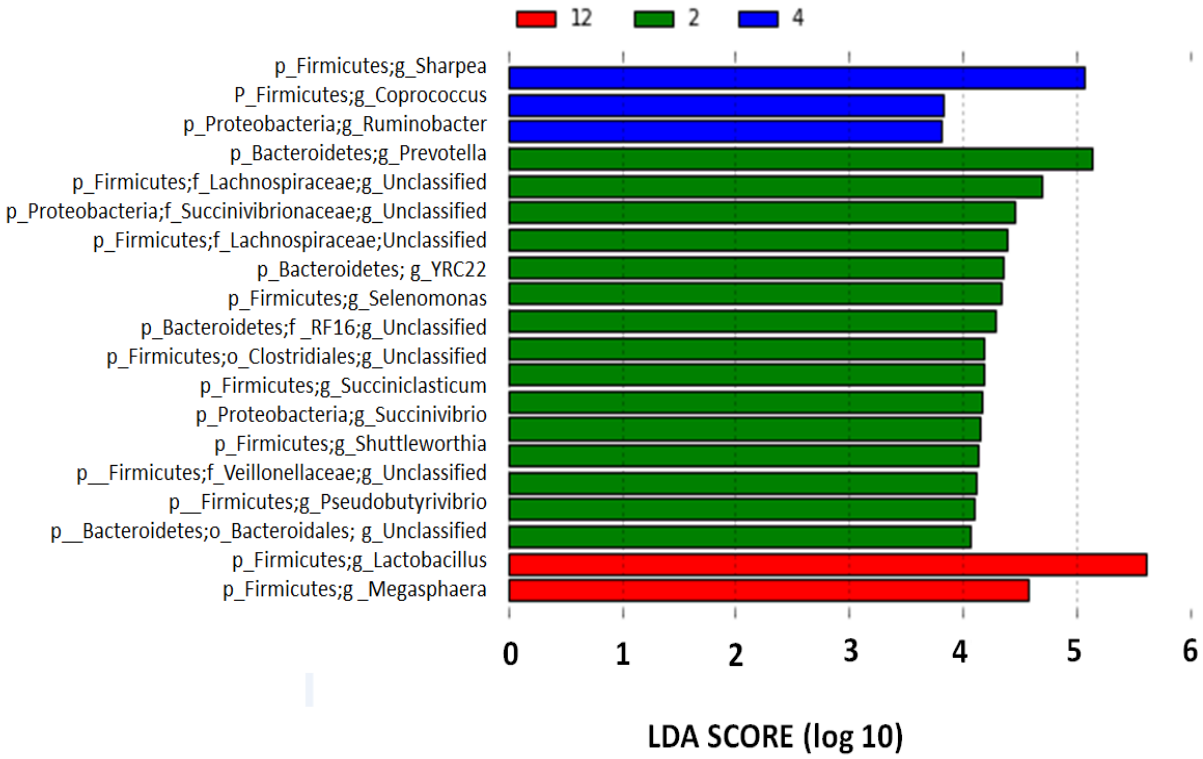


Figure 3.6. Differentially abundant genera associated with barley grain after 2, 4, and 12 h incubation in the rumen of heifers fed a low grain diet from LEfSe analysis (n=6 per time point per treatment). With a log LDA score above 2.00. Lower case letter before taxonomy indicates phylum (p_), family (f_), or genus (g_).

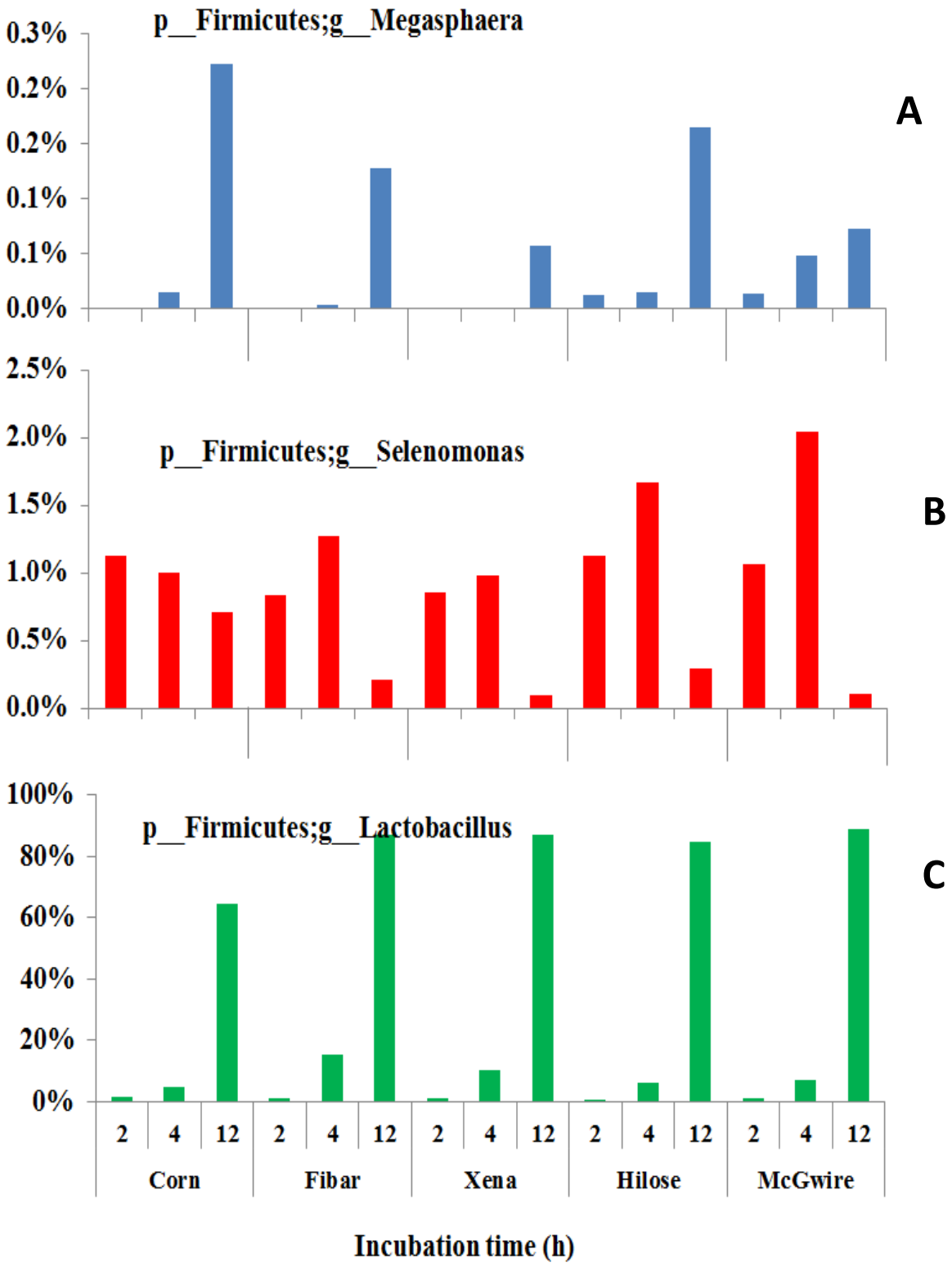


Figure 3.7. The lactic acid-utilizing bacteria (A and B) with *Lactobacillus* (C) in heifers fed low grain diet by different time point (n=6 per time point per treatment). Lower case letter before taxonomy indicates phylum (p_) or genus (g_).

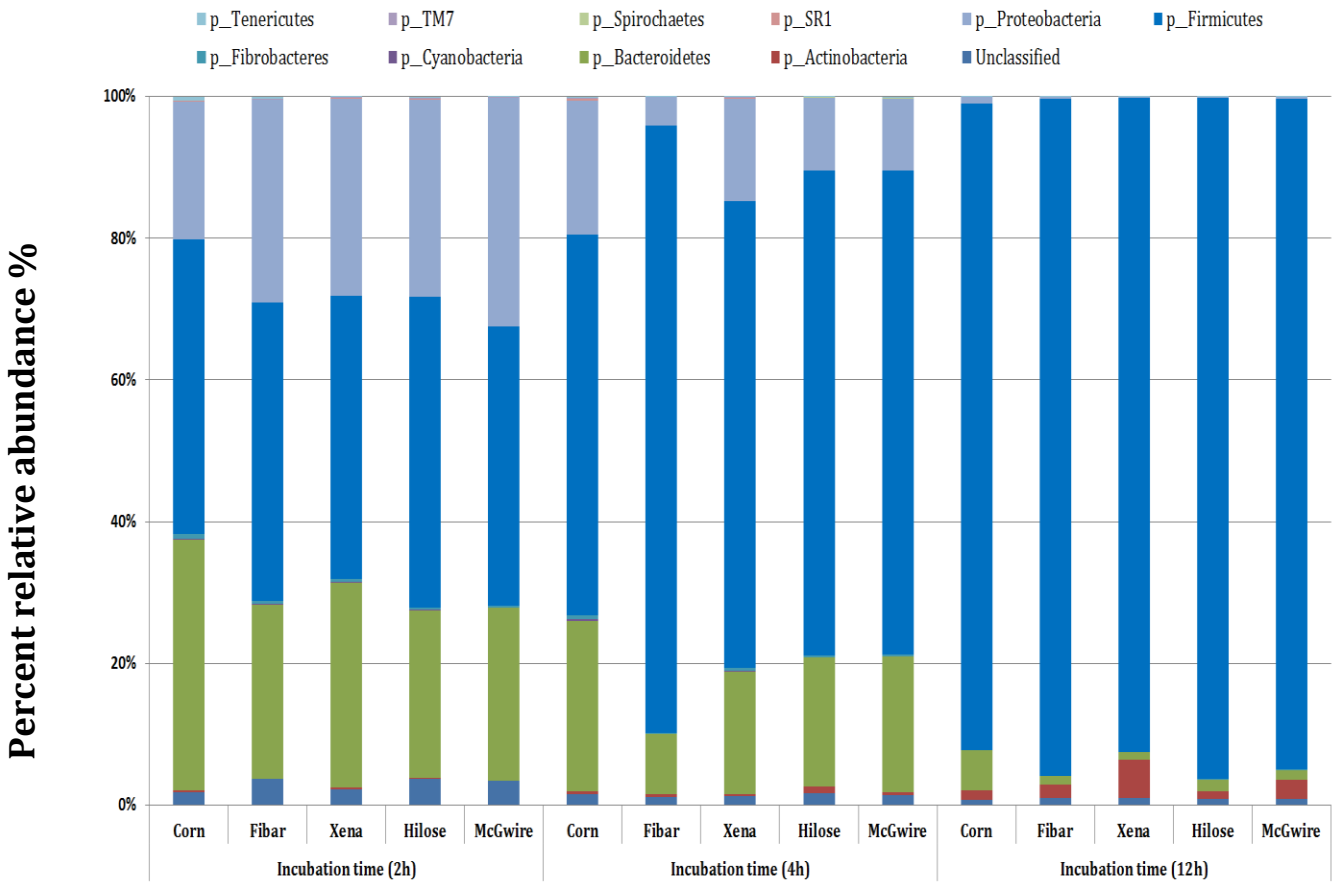


Figure 3.8. Phylum level classification of the biofilm bacterial community composition associated with corn and four barley varieties after 2, 4, and 12 h of incubation in the rumen of heifers fed a low grain diet(n=6 per time point per treatment). Lower case letter before taxonomy indicates phylum (p_).

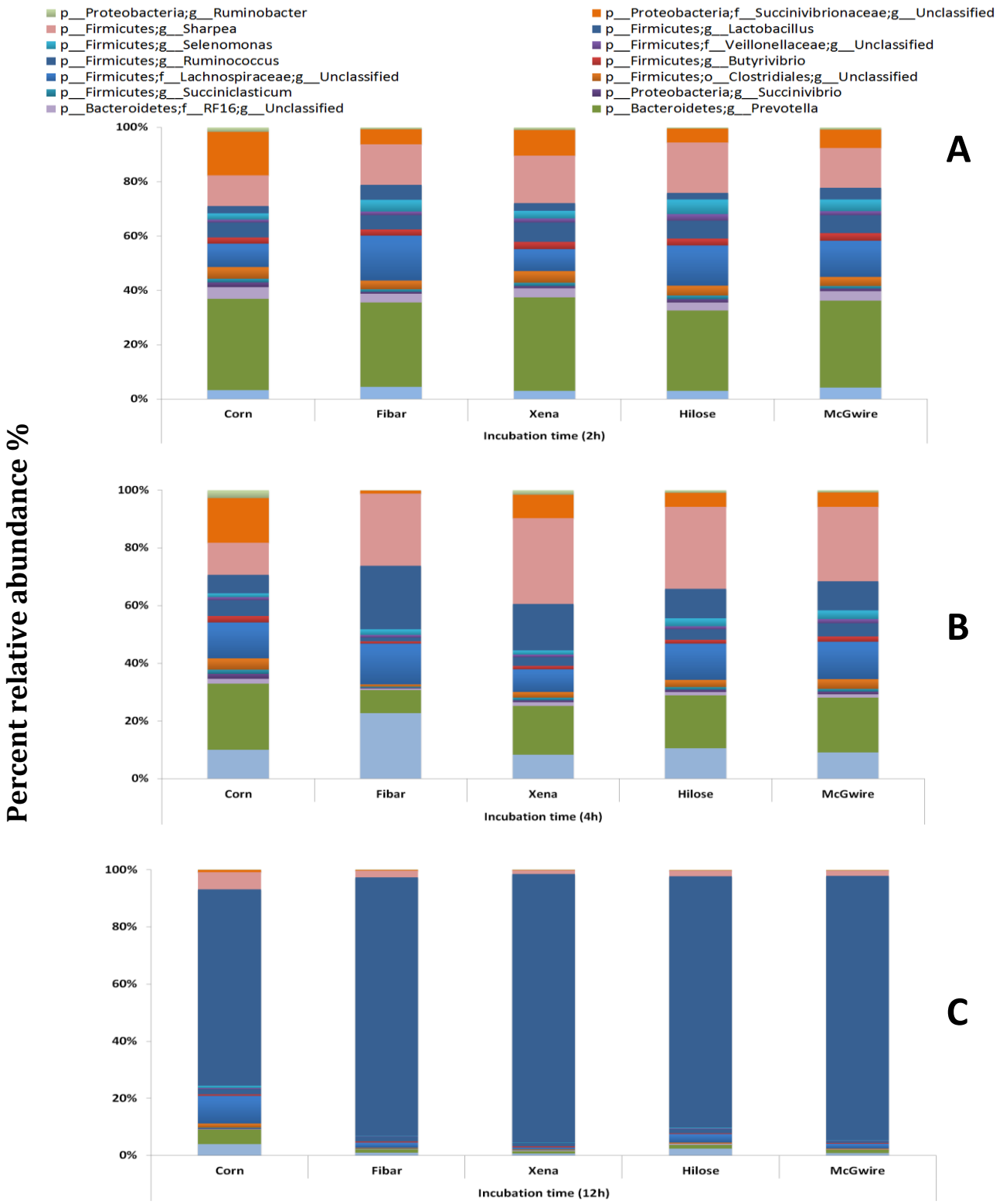


Figure 3.9. Fifteen most abundant genera within the bacterial biofilm community associated with corn and four barley varieties after incubation in the rumen of heifers fed a low grain diet(n=6 per time point per treatment). Lower case letter before taxonomy indicates phylum (p_), family (f_), order (o_) or genus (g_).

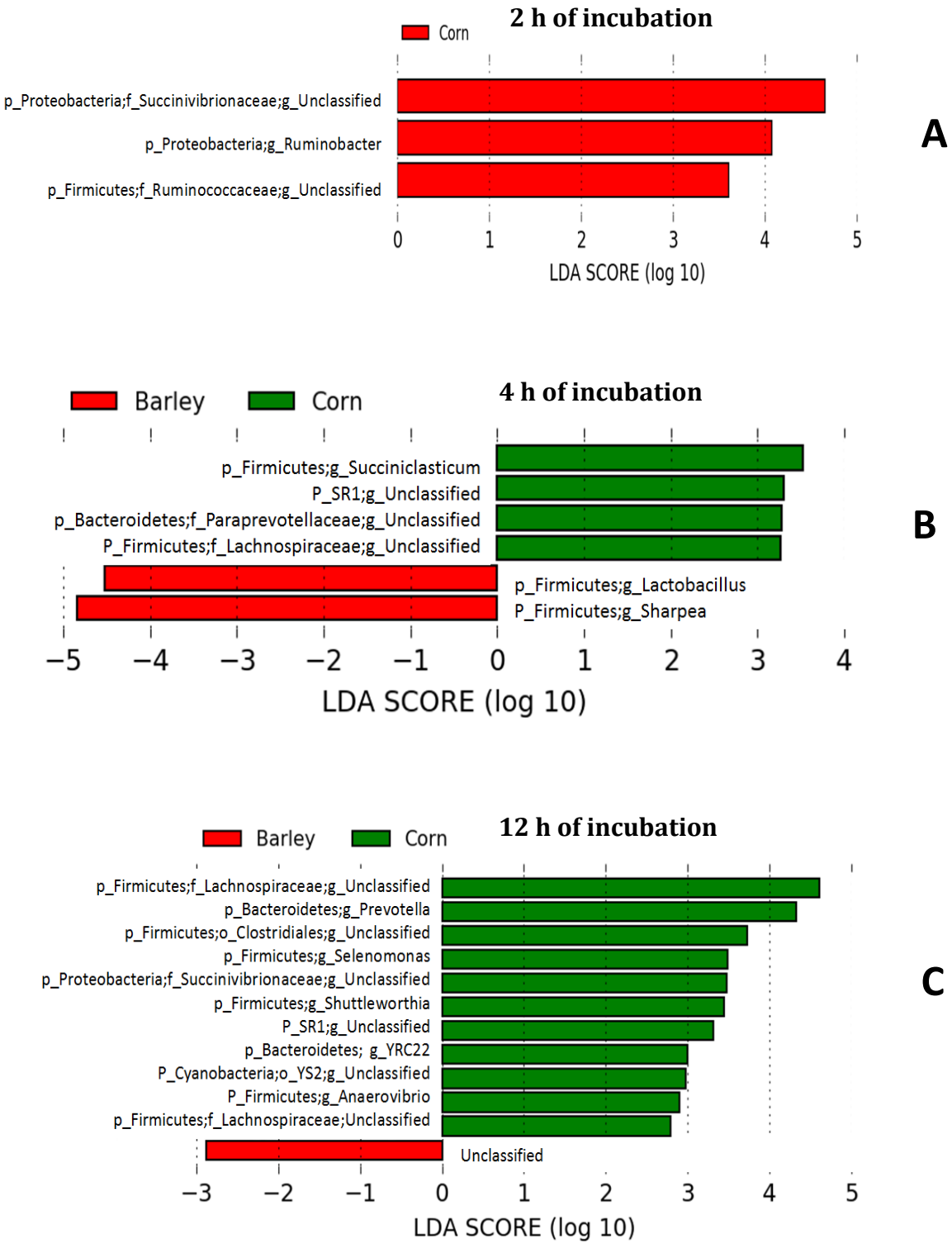


Figure 3.10. Comparison of corn and barley grain associated biofilms illustrating an increase in the abundance select genera after 2, 4, and 12 h of incubation in the rumen of heifers fed a low grain diet from LEfSe analysis (n=6 per time point per treatment). With a log LDA score above 2.00. Lower case letter before taxonomy indicates phylum (p_), family (f_), order (o_) or genus (g_).

3.3.4 Differences in particle-associated bacteria (PAB) between corn and the four barley varieties when heifers were fed high grain diet

With the high grain diet, a total of 395,972 raw sequences were generated. After trimming, quality filtering and chimera checking, a total of 56,814 sequences remained; with an average length of 400 bp. The reads were clustered into 815 OTUs, and their representative sequences were used in taxonomic analysis. The 815 OTUs were classified into 7 phyla, 29 families, 53 genera and 1 unclassified group. Species diversity, richness and evenness were calculated as described above. No significant differences in Chao1 (species richness), observed species (observed OTUs), PD whole tree, or Shannon's diversity index were observed between corn and the four barley varieties (Figure 3.11). Based on a Jackknife similarity plot at 97% identity, estimated hierarchical clustering of a corn and barley samples is illustrated in Figure 3.12. Our results showed that the PCoA in heifers fed high grain diet produced results that were similar to that observed when the heifers were fed a low grain diet. Bacterial communities clustered more by individual heifer at early incubation times (2 and 4 h), but after 12 h biofilms had matured and were similar between animals (Figure 3.13). In heifers fed the high grain diet, the bacterial composition of the biofilm was also affected by incubation time (Figure 3.14). After 2 h of incubation, *Prevotella*, unclassified *Succinivibrionaceae*, and unclassified *Ruminococcaceae* were higher (P<0.05) in barley grain than after 4 and 12 h of incubation, and *Lactobacillus* were higher (P<0.05) after 12 h of incubation.

Overall, 8 phyla were found to be associated with the biofilms on corn and barley in heifers fed the high grain diet (Figure 3.15). At 2 h of incubation, *Bacteroidetes* and *Firmicutes* were the predominant phyla. After 4 h of incubation, *Bacterodietes* and *Firmicutes* were still the most dominant phyla. *Firmicutes* was the most dominant phyla l After 12 h of incubation,

Firmicutes was the most dominant phyla present. A total of 53 genera were identified across all samples, with the fifteen most abundant genera displayed in Figure 3.16. After 2 and 4 h of incubation, *Prevotella* was most abundant in corn and barley grains, followed by unclassified *Veillonellaceae*. Whereas after 12 h of incubation, *Lactobcillus* was higher in corn (44.5%), Fibar (57.0%), Xena (45.0%), Hilose (45.1%), and McGwire (31.5%) than after 2 or 4 h of incubation (Figure 3.16). Compared to barley after 2 h of incubation, corn had higher unclassified *Ruminococcaceae* and *Fibrobacter* than barley grain (Figure 3.17A). After 4 h of incubation, CF231 was higher in corn than barley (Figure 3.17B).

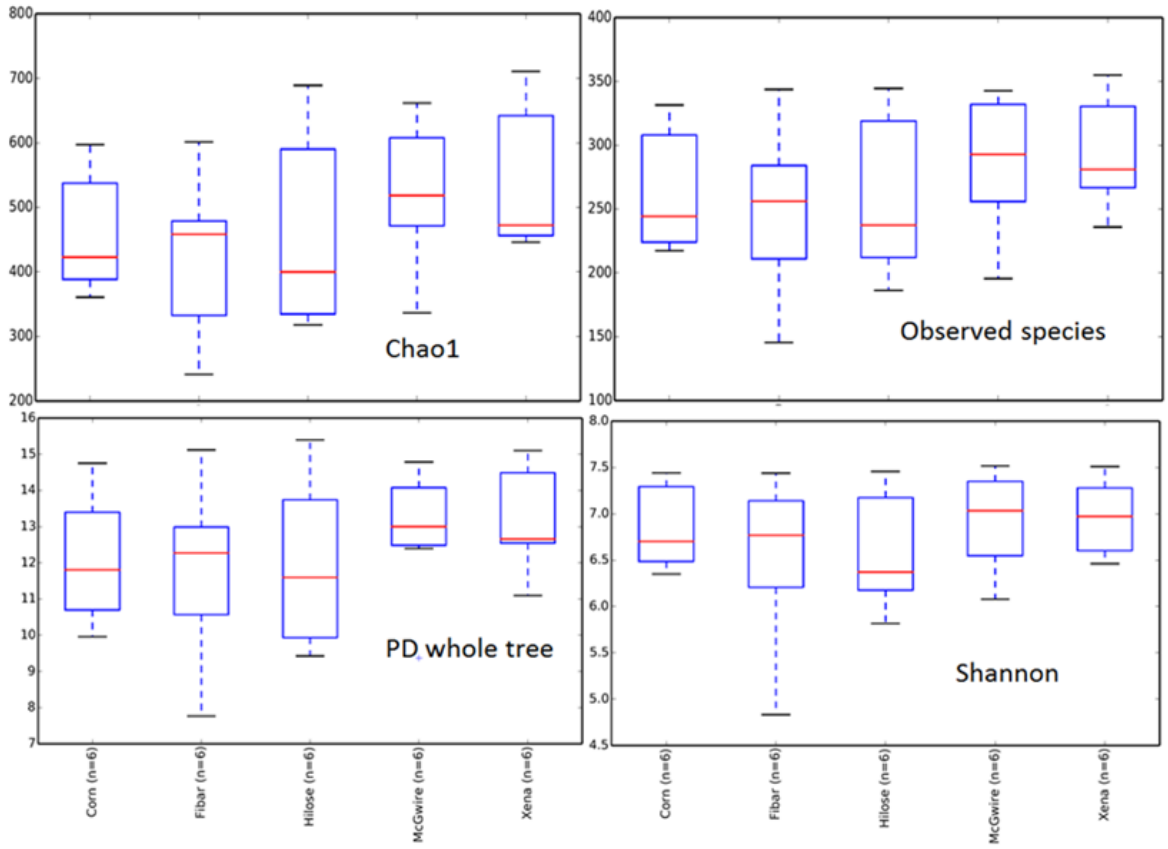


Figure 3.11. Alpha diversity measures of Chao1, Observed species, PD whole tree, and Shannon diversity index associated with biofilms on the surface of corn (n=6) and barley (n=6) per treatment in the rumen of heifers fed a high grain diet.

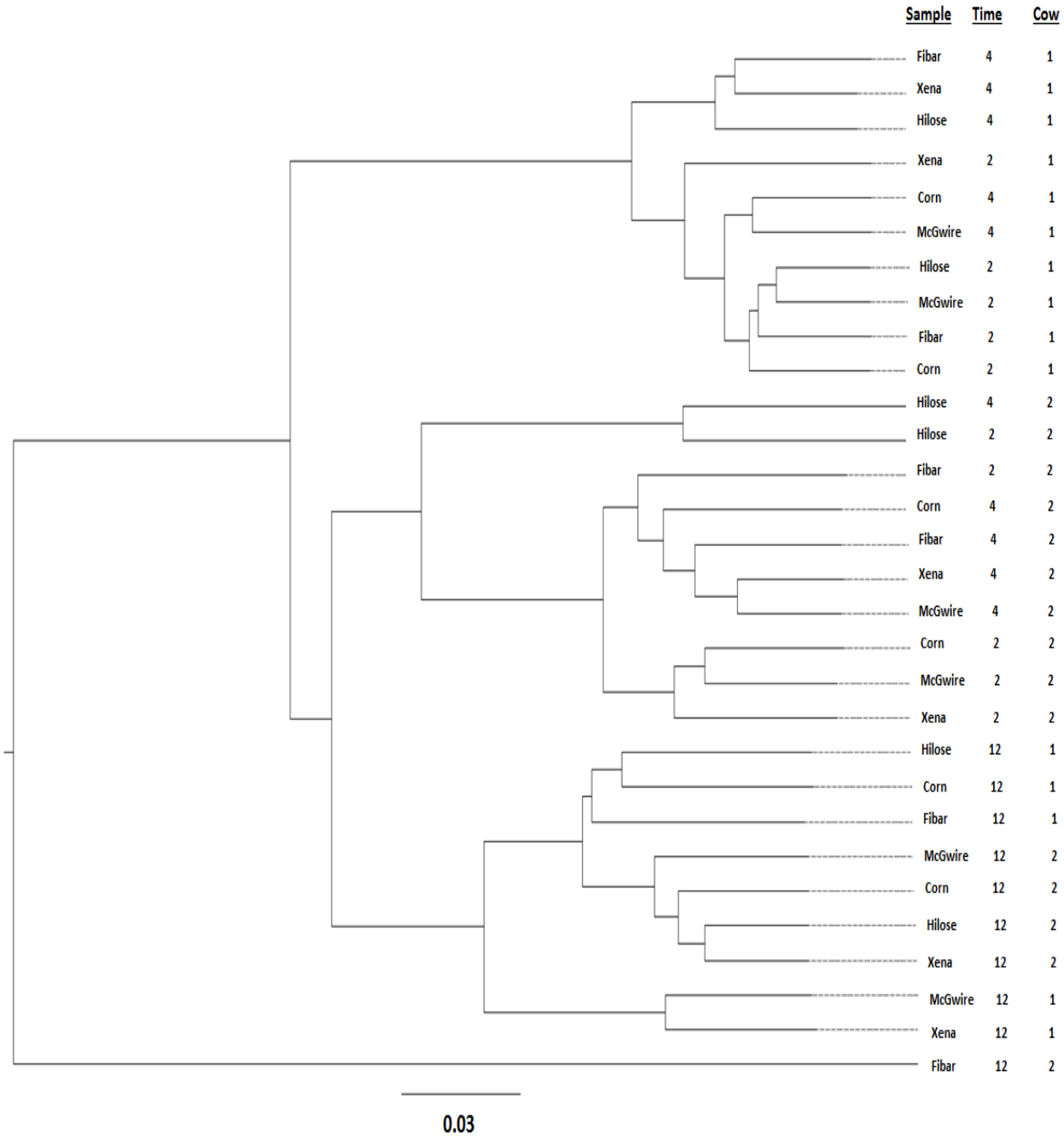


Figure 3.12. Dendrogram shows the relationships among the bacterial communities when heifers were fed high grain diet (n=6 per time point per treatment). The dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm and Bray-Curtis calculation for determining the distance between communities.

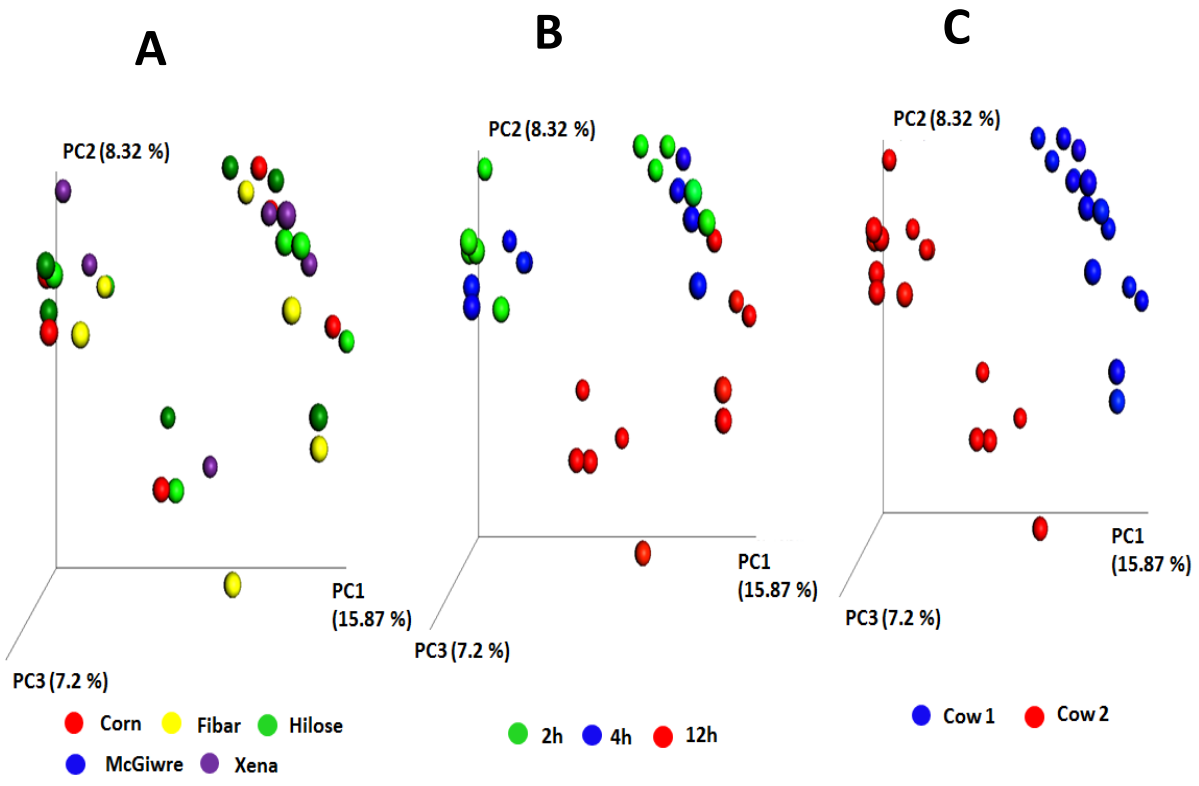


Figure 3.13. Principle coordinate analysis (PCoA) plots of the unweighted UniFrac distances for the bacterial biofilm associated with corn and four barley varieties in heifers fed high grain diet A) a corn and four barley varieties, B) sampling time and C) sampling animal (n=6 per time point per treatment).

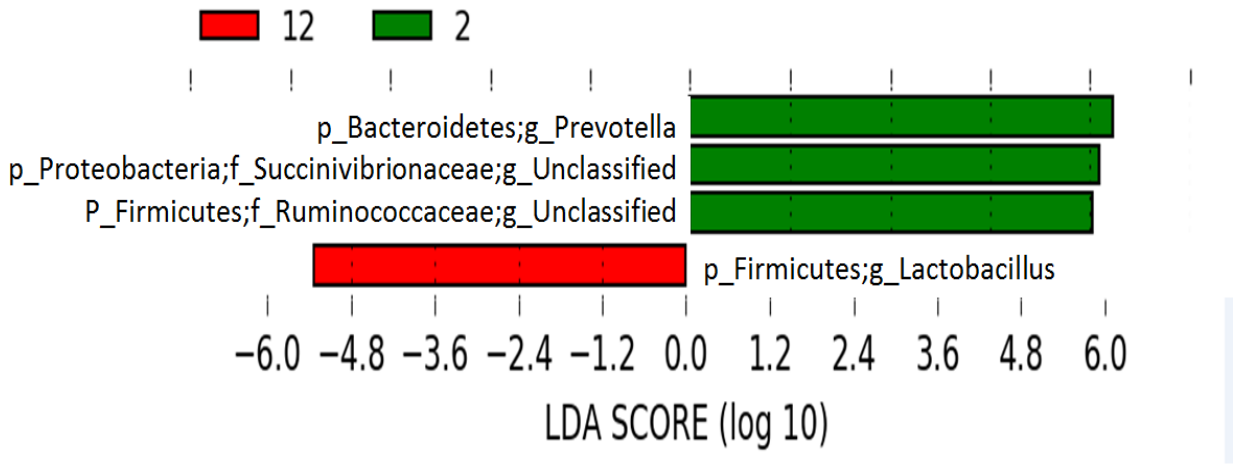


Figure 3.14. LEfSe results showed a statistically significant increase in the abundance in barley grain at genus level at 2, 4, and 12 h incubation when heifers were fed high grain diet from LEfSe analysis (n=6 per time point per treatment). With a log LDA score above 2.00. Lower case letter before taxonomy indicates phylum (p_), family (f_), or genus (g_).

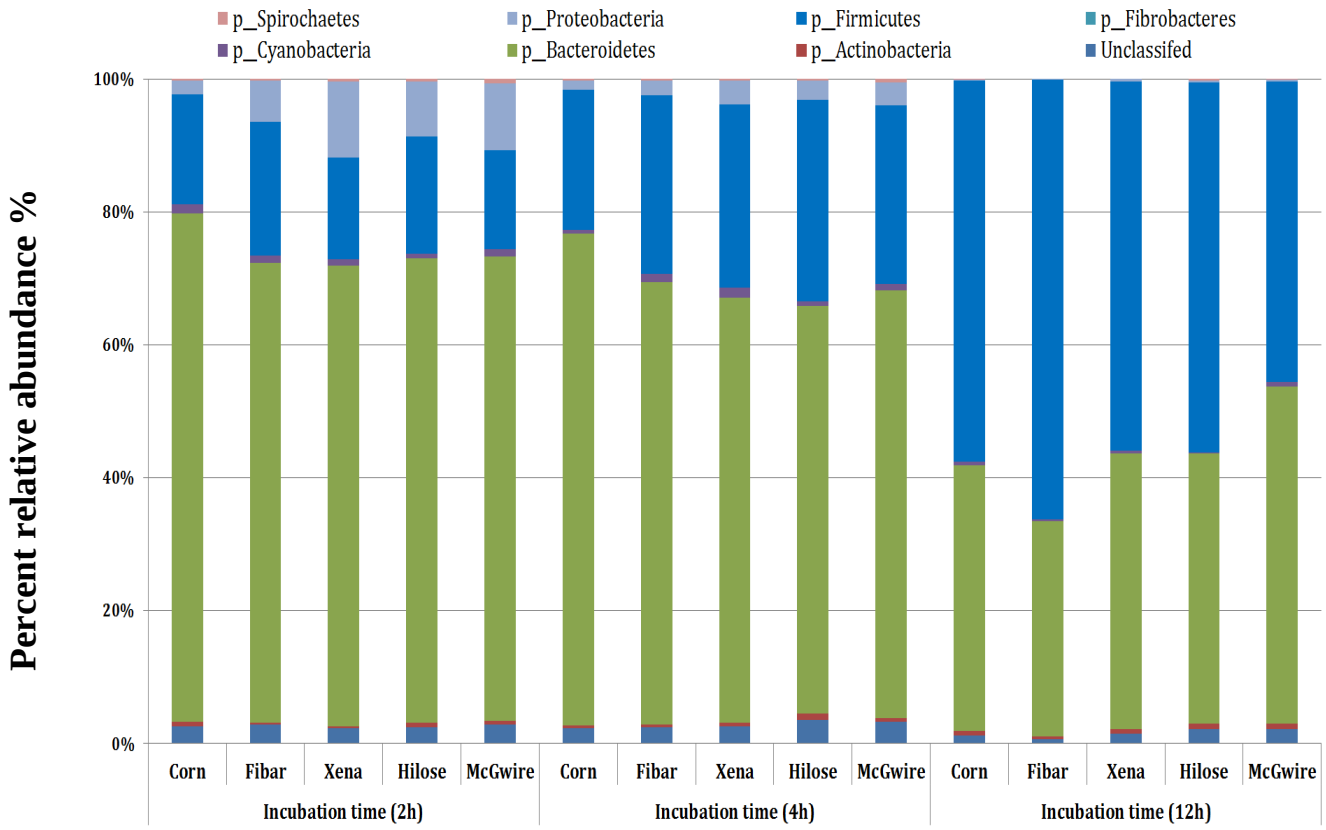


Figure 3.15. Phylum level classification of the biofilm bacterial community composition associated with corn and four barley varieties after 2, 4, and 12 h of incubation in the rumen of heifers fed a high grain diet (n=6 per time point per treatment). Lower case letter before taxonomy indicates phylum (p_).

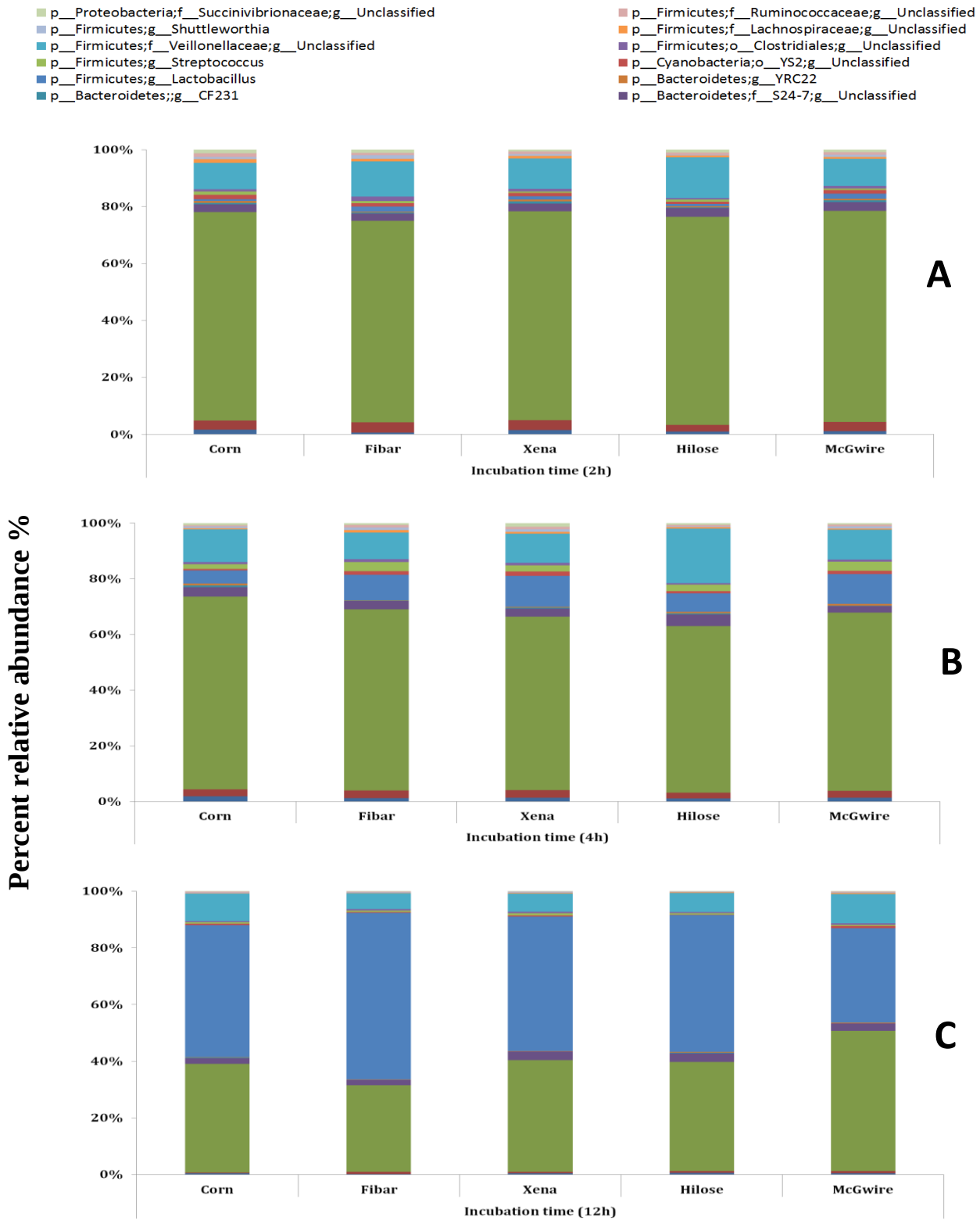


Figure 3.16. Fifteen most abundant genera level classification of the bacterial community composition in a corn and four barley varieties when heifers were fed high grain diet (n=6 per time point per treatment). Lower case letter before taxonomy indicates phylum (p_), family (f_), order (o_) or genus (g_).

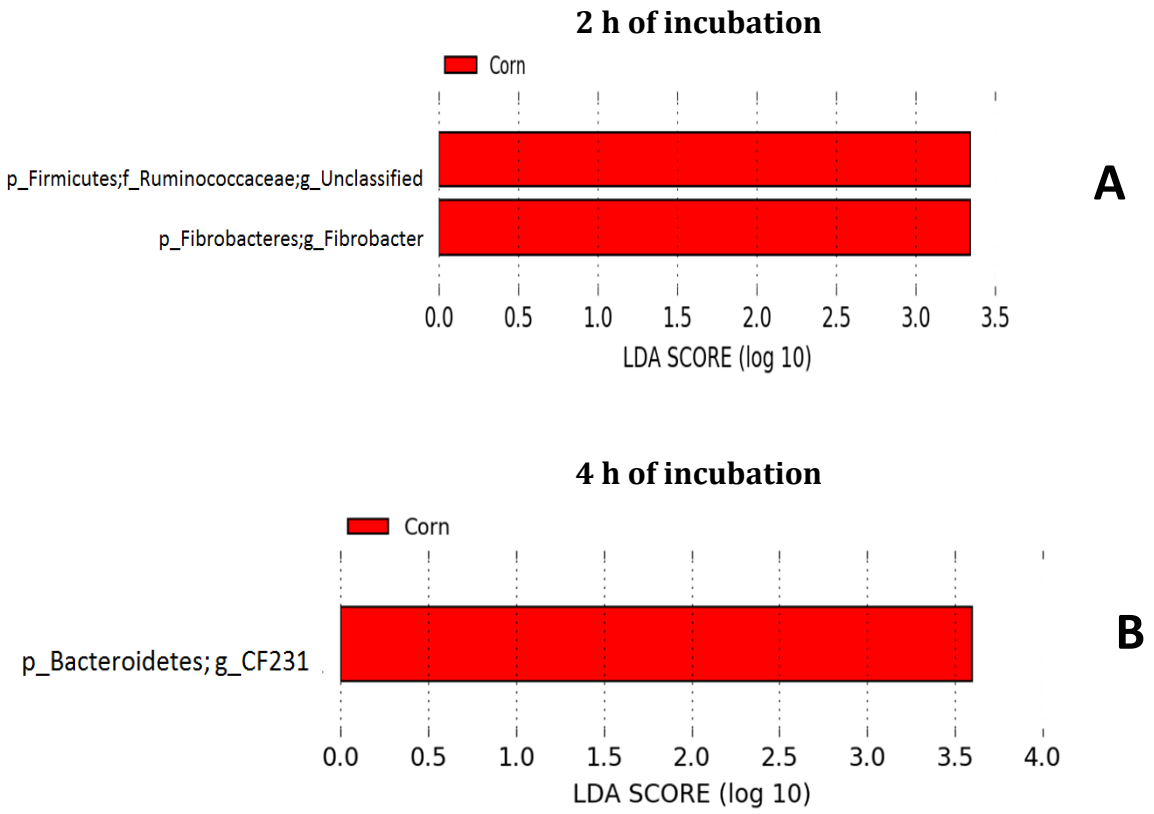


Figure 3.17. Comparison of corn and barley grain showed a statistically significant increase in the abundance at genus level at 2, 4, and 12 h of incubation when heifers were fed high grain diet from LEfSe analysis (n=6 per time point per treatment). With a log LDA score above 2.00. Lower case letter before taxonomy indicates phylum (p_) or genus (g_).

3.3.5 Bacterial morphologies associated with barley grain

After 2 h of incubation, SEM images showed initial colonization of starch granules by rumen bacteria, but digestive pits on the surface of starch granules were not apparent (arrows, Figure 3.18A and B). By 4 h of incubation the formation of rich biofilms and along with digestive pits on the surface of starch granules were evident (arrows, Figure 3.18C and D). After 12 h of incubation in the rumen, the protein matrix of starch granules was also undergoing invasion by bacteria and penetration of starch granules by starch-digesting bacteria was commonly observed (Figure 3.18E and F).

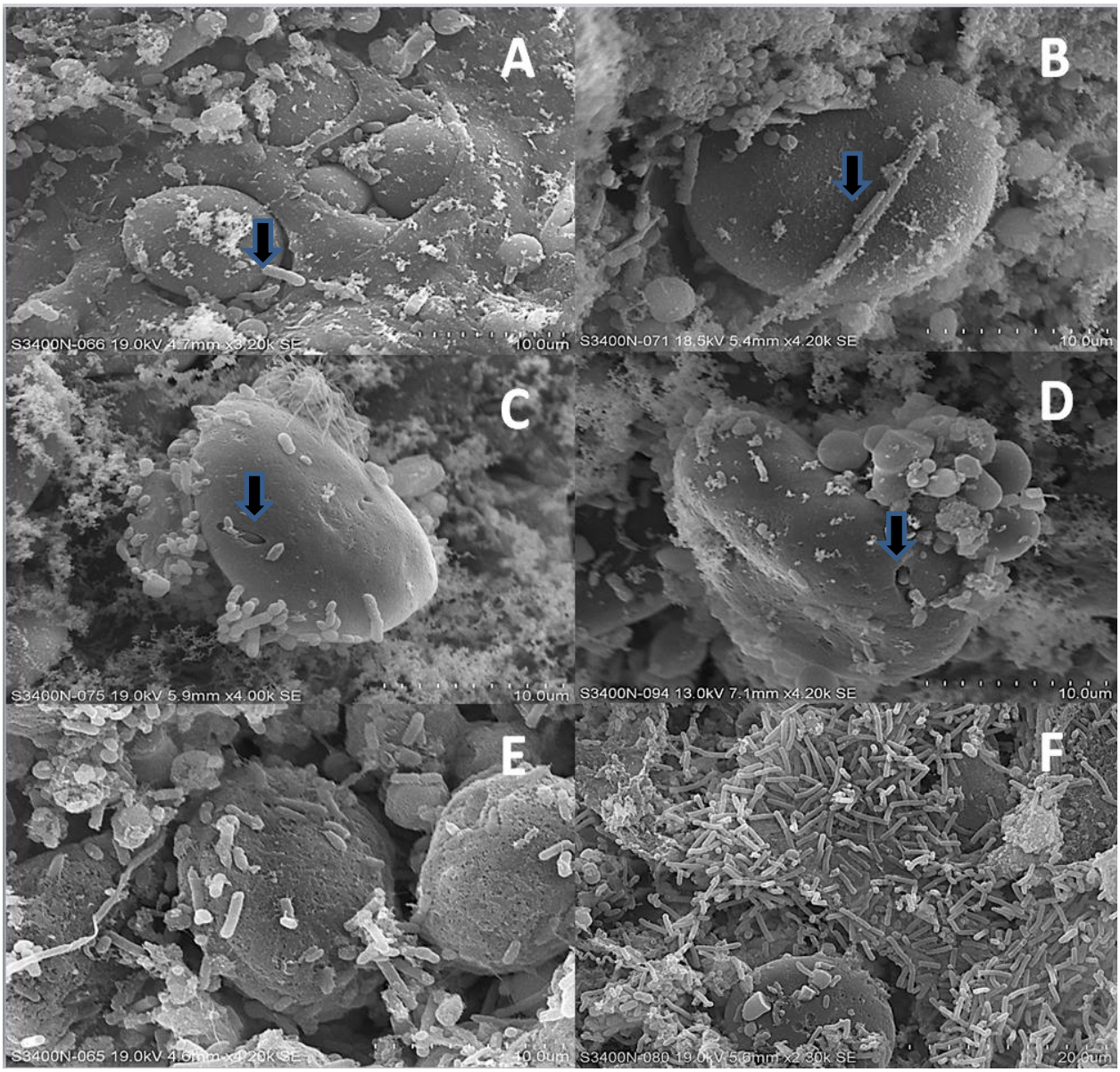


Figure 3.18. Rumen bacteria on grain surface by Scanning Electron Microscopy (SEM) at 2, 4 and 12 h of incubation. (A) Fibar after 2 h of incubation (B) McGwire after 2 h of incubation (C) Fibar after 4 h of incubation (D) McGwire 4 h of incubation (E) Hilose after 12 h of incubation (F) Fibar after 12 h of incubation

3.4. Discussion

3.4.1 Digestibility after *in situ* incubation of different barley varieties when heifers were fed low and high grain diets

It has been proposed that biofilms are responsible for 80% of the endoglucanase activity, 70% of the amylase activity (Minato et al., 1966), and 75% of the protease activity (Brock et al., 1982) in the rumen. Consequently, biofilms are associated with the majority of ruminal feed digestion (McAllister et al., 1994). The aim of our study was to compare the rumen digestibility of four barley varieties and to characterize the biofilms associated with this digestion using next generation sequencing methods in heifers fed low and high grain diets. We hypothesized that biofilm formation and bacterial composition would be affected by grain structure and composition, resulting in differences in *in situ* digestibility of barley varieties and corn within the rumen.

Our study showed that the normal barley varieties of CDC McGwire and Xena, which generally contain an amylose:amylopectin ratio of 1:3, exhibited a higher DM digestibility as compared to high amylose (CDC Hilose) and high amylopectin (CDC Fibar content) barleys or corn (Table 3.3). Similarly, Stevnebø et al., (2009) showed that low amylose content barley varieties had a greater effective kd (0.148/h) compared to normal amylose (0.115/h) and high amylose (0.102/h) barley varieties. However, studies comparing the impact of the amylose/amylopectin ratio on starch digestibility in the rumen are still limited and inconsistent. Philippeau et al. (1998) concluded that ruminal starch degradability was not related to amylose content in corn starch, as measured by the *in situ* bag technique. Interestingly, in our study, corn had a lower DM digestibility in both diets as compared to barley. According to McAllister et al. (1993), differences in ruminal starch digestibility between corn and barley grain are related to

differences in the composition and structure of the endosperm rather than of starch granules. Moreover, other studies showed that the reduced digestibility of starch in corn was attributable to the protein matrix surrounding starch granules (Weurding et al., 2001). In corn, starch granules may be so tightly associated with the protein matrix (Philippeau et al., 2000; McAllister and Ribeiro, 2013), that starch resists degradation by rumen bacteria (McAllister et al., 1993; Philippeau et al., 2000), resulting in a lower rate of digestibility for coarsely ground corn as compared to barley.

Even though our study observed differences in the digestibility of the barley varieties in both diets, we did not demonstrate clear differences in the biofilm bacterial communities associated with different barley varieties in heifers fed either low or high barley grain diets. However, our study showed changes in the microbiota over time (i.e 2, 4 and 12 h of incubation) regardless of diet. Interestingly, biofilms exhibited the different bacterial diversity between corn and barley grain at 2, 4, and 12 h of incubation in heifers fed low and high grain diets as well. This is probably a result of structural differences such as protein matrix of the grain (i.e. corn versus barley) affecting surface biofilm development rather than differences in starch composition (i.e. between barley varieties). However, the bacterial communities were higher in bacterial diversity and complexity in heifers fed high forage diet than high grain fed heifers. Generally, biofilms are formed by dominant bacteria and evolve with incubation time, possibly in relation to changes in the composition of the feed as digestion proceeds.

3.4.2 Temporal microbial colonization of corn and barley grain within the rumen

The biofilms responsible for digestion of feed particles develop overtime, with three successive populations contributing to the biofilm (McAllister et al., 1994; Huws et al., 2016). The first population consists of those loosely associated bacteria that utilize those water soluble

sugars and proteins that are available in the early stages of digestion. This population is followed by more tightly-associated populations that adhere to the surface of plant cell walls and starch granules, contributing to the establishment of a stable biofilm. Finally, a third population integrates into the biofilm, utilizing the fermentation end products produced by the tightly associated population. Huws et al. (2013) showed that the primary colonizing bacteria were associated with fresh perennial rye grass within 1 to 2 h after its introduction into the rumen. Secondary colonizers were shown to join the biofilm of fresh perennial rygrass within 4 to 8 h. Interestingly, using denaturing gradient gel electrophoresis , rumen biofilms associated with Chinese wild rye were shown to continue to evolve even after 6 and 12 h of incubation in the rumen (Sun et al., 2008). These studies support our microscopy data (Figure 3.18), which showed that at 2 h incubation, bacteria initiated colonization of starch granules, but mature biofilms had not formed and bacterial invasion of starch granules and the protein matrix had not yet occurred. At 4 h of incubation, bacterial penetration of starch granules was apparent, and rich biofilms were visible on the surface of both starch granules and the protein matrix after 12 h of incubation. Thus our study demonstrated that colonization of corn and barley varieties by rumen bacteria with primary (2 h of incubation) colonizers, secondary (4 h of incubation) colonizers, and tertiary biofilm development occurring within 12 h of incubation.

3.4.3 Temporal microbial colonization between corn and the four barley varieties in heifers fed low grain diet

Even though the bacterial communities in biofilms associated with the four barley varieties did not differ (i. e. primary, secondary colonization, and tertiary biofilms), the bacterial composition of the biofilms did change dramatically over time (Figure 3.6). Our study showed the abundance of Bacteroidetes (*Prevotella,* YRC22, Unclassified RF16, and Unclassified

Bacteroidales), Firmicutes (Unclassified *Lachnospiraceae, Selenomanas*, Unclassified *Clostridiales, Succiniclasticum, Shuttleworthia*, Unclassified *Veillonellaceae,* and *Pseudobutyrivibrio*), and Proteobacteria (Unclassified *Succinivibrionaceae* and *Succinivibrio*) increased (P<0.05) during the primary biofilm formation (Figure 3.6). Our study observed that the primary biofilm formation has a significantly higher bacterial diversity with fibre, starch, and protein-digesting bacteria compared to the secondary and tertiary events in biofilm formation. This may reflect that there is a rich variety of soluble and insoluble nutrients available for digestion during the early stages of biofilm formation, resulting in a greater diversity of bacteria participating as digestion proceeds. As digestion proceeds, soluble nutrients are exhausted and the biofilm becomes more mature and less compositionally diverse (Watnick and Koltr, 2000). Similarly, Huws et al. (2016) study also showed that a higher bacterial diversity associated with fresh perennial ryegrass after 1 to 2 h of incubation in the rumen as compared to 4 to 6 h of incubation. Compared to barley, primary biofilm formation in corn had higher unclassified *Succinivibrionaceae, Ruminobacter,* and unclassified *Ruminococcaceae,* a response that may reflect the higher level of starch in corn. According to Huws et al. (2016) primary colonizing bacteria associate first with soluble nutrients, and secondary colonizing bacteria are adept at digesting starch and plant cell walls.

During the secondary colonization of barley grain, *Sharpea*, *Coprococcus*, and *Ruminobacter* increased (P<0.05) within the biofilm. These bacteria are associated with starch digestion in the rumen. As soluble carbohydrates are utilized, the digestion of starch likely intensifies (Figure 3.6). Digestion is also likely enhanced from primary colonizers degrading physical barriers and increasing access to starch, for use by seconday colonizers. Comparison of the secondary colonizers found that four genera (*Succiniclasticum*, unclassified

SR1, Unclassified *Paraprevotellaceae*, and Unclassified *Lachnospiraceae*) were more abundant in biofilms associated with corn than barley (Figure 3.10B).

Whereas at this same point, *Lactobacillus*, and *Sharpea* were more abundant in biofilms associated with barley than corn (Figure 3.10B). The probable explanation is that corn and barley grain have different starch structure with in the protein matrix. The starch endosperm of corn tightly associated with the protein matrix, which resists degradation by ruminal bacteria (Philippeau et al., 2000). Whereas, the starch of barley grain is loosely associated with the protein matrix easily exposed and digested by ruminal bacteria. Therefore, these different starch structures affected the bacterial communities and also the digestibility in the rumen.

In general, tertiary biofilms became less diverse over time within the rumen and selected bacteria were richer at the tertiary biofilm. The study showed that *Lactobacillus* and *Megasphaera* increased in the tertiary biofilm associated with barley grain during primary and secondary colonization (Figure 3.6). Generally, *Lactobacillus* is most often associated with acidosis in the rumen (SATO, 2016) due to producing large amounts of lactic acid (Hernandez et al., 2007). However, surprisingly, we observed that *Lactobacillus* increased and account for a significant amount of the bacterial population adhered to grains, even when heifers were fed low grain diets. We also found that lactic acid-utilizing bacteria (i.e. *Selenomonas* and *Megasphaera*) increased, which increased *Selenomonas* at the secondary colonization and *Megasphaera* at the tertiary biofilm (Figure 3.7A and B). These bacteria have an important role in preventing the accumulation of lactic acid under typical conditions of starch digestion in the rumen (Castillo-Gonzaleza et al., 2014). Thus the increase in *Lactobacillus* observed, and the likely increase in lactic acid production was offset by an increase in lactic acid utilizers such as *Selenomonas* and *Megashereae* (Figure 3.7). This suggests that *Lactobacillus* is active in starch

digestion even when acidosis is not taking place. This is likely because of the lactic acid utilizers that utilize lactic acid as quickly as *Lactobacillus* and thereby keep the concentration of lactic acid in the rumen low. Counotte et al. (1981) also suggested that *Megasphaera elsdenii* is the main species responsible for lactic acid metabolism; thus, it has an important role in the prevention of acidosis during the adaptation period when ruminants are fed diets high in concentrate.

Unclassified *Lachnospiraceae, Prevotella*, unclassified *Clostridiales,* unclassified S*uccinivibrionaceae, Shuttleworthia,* unclassified SR1, *YRC22*, unclassified YS2, and *Anaerovibrio* were more dominant in tertiary biofilms associated with corn than with barley (Figure 3.10C). The protein and starch association of the grain can affect the degradation and the bacterial community in the rumen. As shown in Table 3.3 at the low grain diet, the starch and protein degradability of corn was lower than barley because the starch of corn has a high content of protein resistant to enzymatic and physical degradation, resulting in a low degradability (Kotarski et al., 1992). This suggests that the different nature of the protein matrix or other chemical constituents such as lipid may result in greater bacterial diversity in mature biofilms in corn than the biofilms associated with barley grain. Likewise, *Prevotella* was higher in corn than barley because *Prevotella* is well recognized for their proteolytic capacity (Wallace et al., 1995) due to the possession of numerous peptidase enzymes (Wallace et al., 1997). The reason for increased abundance of unclassified *Clostridiales* during the tertiary biofilm in corn is unclear, but these genera could associate with the protein matrix in corn. Similarly, Poppi and Quigley, (2003) showed that the abundance of unclassified *Clostridiales* increased significantly with increasing N intake of cottonseed meal supplement. Another unique genus was *Anaerovibrio* in corn, which is a fat-degrading bacterium in the rumen. This is probably

because corn has higher content lipid than barley grain (Table 3.2). Generally, corn lipids are predominately acyltriglycerides, whereas barley grain contain about 65-78 % nonpolar lipid, 7-13 % galactolipid and 15-26 % phospholipids (Morrison, 1978).

3.4.4 Temporal microbial colonization between corn and the four barley varieties in heifers fed high grain diet

Biofilm bacterial communities associated with corn and barley grain were also influenced by the level of grain that was high grain fed to heifers (Figure 3.12). The three genera that were higher at the primary colonization of barley grain compared to the secondary colonization and tertiary biofilms, which were *Prevotella*, Unclassified *Succinivibrionaceae*, and Unclassified *Ruminococcaceae*. These three genera are associated with starch digestion, and appeared to be more abundant in the high grain diet than the low grain diet. This suggested that high grain diet fed heifers have less bacterial diversity than in heifers fed high forage diet. Specially, the bacteria associated with fibre digestion were less in heifers fed high grain diet at the primary colonization compared to high forage fed heifers. This may depress fibre digestion in heifers fed high grain diet compared to high forage diet, and may be a reflection of the microbial communities.

Interestingly, our data showed that *Prevotella* was the most dominant genus both grain types (i.e. corn vs barley grain) and diets (i.e. high fibre and high grain diets) during the initiation of biofilm formation. This is probably that *Prevotella* is initial colonizer, predominant, and has multiple digestible functions in the rumen. Koike et al. (2003) also showed that *Prevotella* were found to be predominant in the total 16S rRNA sequences retrieved from the particle-associated community in the rumen, and also this genus were involved in the degradation of a fibre and grain (Bekele et al., 2010). Members of this genera are known to possess amylolytic and

proteolytic (Accetto and Avguštin, 2015; Kishi et al., 2015), but also they play an important role in the degradation of protein and in the uptake and fermentation of peptides (Wallace et al., 1993) as well.

In contrast to heifers fed low grain diet, at the mature biofilm formation in heifers fed high grain diet did not showed any differences of bacterial community between corn and barley varieties. This is probably that the biofilms in heifers fed high grain diet had less bacterial diversity, thus the development of biofilms were less complex. Fernando et al. (2010) also showed the bacterial species based on chao1 and ACE presented the significantly higher number of unclassified bacteria in prairie hay-fed animals suggests that the ruminal bacterial diversity in animals on forage-based diets is higher than that in grain-fed animals.

3.5 Conclusions

The development and advancement of molecular techniques that study 16S rRNA gene sequences have identified and characterized bacteria not previously recognized by culture-based studies of rumen microorganisms (Mcsweeney et al., 2006; Kim et al., 2011b). Our study showed that normal barley varieties had a higher ruminal DM, starch, and protein digestibility compared to a higher content of amylose and amylopectin barley types, and also these digestibilities were affected by different starch structure such as corn and barley grain as well. This is probably because corn and barley grain have different starch structure with the protein matrix, this different starch structure could affect the digestibility by rumen bacteria. However, even with these different results in digestion among barley varieties, the bacterial community was not different among barley varieties during different colonization stages such as primary, secondary colonization and mature biofilm in both diets (heifers fed low and high grain diet). However, the bacterial communities did differ depending on the stage of biofilm development.

Our result showed that *Prevotella* was initial attached bacteria because *Prevotella* was predominant genus at the primary colonization with the high fibre and grain diets. The secondary colonizers were affected by primary colonizers. Our study showed that the starch-digesting associated bacteria increased at the secondary colonization due to easily digest the starch endosperm by primary colonizers. Moreover, the lactic-utilizing bacteria increased at the secondary colonization as well. And the mature biofilm stage became less diverse (fewer OTUs), but richer a selected for bacteria such as *Lactobacillus. Lactobacillus* was the most dominant genus in heifers fed low and high grain diets at the mature biofilm stage. In addition, the bacterial communities at the biofilm of corn and barley grain were difference because corn and barley have different starch structure with the protein matrix.

Future strategies should focus the primary colonizers, secondary colonizers, and mature biofilms, their functions for feed particle in the rumen. Therefore, understanding their role may help in the development of methods to manipulate rumen bacteria and enhance animal performance.

4.0 GENERAL DISCUSSION AND CONCLUSION

Barley is Canada's third largest crop and is the predominant grain used in beef production. Several factors can affect the nutritional composition of barley, such as weather, soil quality, and genetic background. Barley varieties have been bred to confer different traits, with some varying in starch and protein composition. This study showed that the nutritional content and rumen effective digestibility of McGwire, Xena, Fibar, and Hilose barley varieties differ. For example, McGwire and Xena had the highest DM digestibility, followed by Fibar and Hilose. Previous studies characterizing the bacteria involved in grain digestion were mostly based on traditional culturing techniques. Newer technologies that are DNA-based, such as next generation sequencing, allow for greater depth of analysis of microbial communities. The objectives of this thesis were therefore to compare digestibility of several barley grain varieties, and corn as a control, and to evaluate the adherent bacterial communities involved in their digestion. It was hypothesized that differences in composition would result in differences in the bacterial microbiome associated with the digestion of the examined barley varieties.

The first objective was to compare ruminal digestibility of four barley varieties in heifers fed either high fibre or high grain diets. The starch composition and *in situ* DMD varied between the four barley varieties, with McGwire having the highest DM and starch disappearance rate of both of these fractions. Several studies have shown that the amylose/amylopectin ratio of grain is negatively correlated with starch digestion (StevnebØ et al 2009; Knowles et al 2012). This supports our findings as generally; the effective degradability of normal starch barley grain (McGwire and Xena) was higher than high amylose (Hilose) barley, regardless of the type of diet fed to heifers. We also observed that corn had lower DM, starch and protein digestibility than barley. According to McAllister et al. (1993), differences in ruminal starch digestibility between

corn and barley grain are related to the structural components within the endosperm. Moreover, other studies showed that digestibility is influenced by the protein matrix surrounding starch granules (Weurding et al., 2001; Baldwin, 2001) and that amylose-lipid interactions may also influence starch degradation (Vasanthan and Bhatty, 1996). Thus overall, we showed that variation in digestibility occurred between barley varieties, with the amylose/amylopectin ratio influencing starch digestion in the rumen. In addition, when compared to corn, digestion was greater for all barley varieties, and was likely due to the protein matrix of corn impeding digestion.

The second objective of this study was to characterize the biofilms during digestion of grains by examining colonized microbiomes using scanning electron microscopy and 454 sequencing of the 16S rRNA gene extracted from these biofilms. Although the bacterial microbiota of corn was different from barley, no differences were observed among the barley varieties. This suggested that the variation in digestibilities among barley varieties was a result of the chemical composition of the grains (e.g. high amylose in Hilose reduced digestion), and not the bacteria associated with digestion. However, there was a clear progression of colonization of barley over time with changes in the microbiota as the biofilm matured.

When fed the low grain diet, primary colonization (2 h of incubation) of barley grains produced bacterial communities that were highly diverse. This was likely reflection of the rich variety of soluble and insoluble nutrients that were available for digestion during the early stages of biofilm formation. *Prevotella* was most abundant genus present and has previously been reported to be a predominant initial colonizer of high forage and grain diets, a result that is likely a reflection of the diverse metabolic capacity of the species within this genus (Jousset et al., 2017). Most secondary colonizers (4 h) were bacteria associated with starch digestion. It is

likely that initial digestion by primary colonizers reduced the variety of nutrients available and also exposed starch to bacterial colonization through the digestion of beta-glucan cell walls within the endosperm. This allowed starch-utilizing bacteria to colonize the endosperm surface by 4 h of incubation. The tertiary biofilm, which represented a mature microbiota, favored the establishment of lactic-acid producing bacteria. *Lactobacillus* was the most abundant genera after 12 h of incubation. Although lactic acid concentration was not measured, most *lactobacilli* produce lactic acid as a fermentation end product. It may be that accumulation of lactic acid in the rumen is limited as a result of the metabolism of lactic acid by lactic acid utilizers such as *Megasphera*, which also increased after 12 h of incubation.

When fed a high grain diet, the primary colonizing bacteria were less diverse than in heifers fed the low grain diet. This was probably a reflection of the bacterial population in heifers fed high grain diet being predominantly starch-digesting bacteria in the rumen as the composition of the microbiome in the rumen is heavily affected by diet. In contrast to heifers fed the low grain diet, the bacterial community associated with the tertiary biofilm in heifers fed the high grain diet did not differ among corn or barley varieties. This is probably a reflection of the biofilms in heifers fed the high grain diet having less bacterial diversity and as a result less complex biofilms. Similarly, Ousama et al., (2017) showed that the diversity of the rumen bacterial community was reduced when rumen-cannulated lactating Holstein cows were switched from a high forage (77:23, forage: concentrate) to high grain diet (49:51, forage: concentrate).

The main strength of this study was using a combination of digestibility, electron microscopy, and NGS data to evaluate the ruminal bacterial microbiota involved in starch digestion. By combining these approaches, a clear progression of the digestion of barley and corn

substrate was achieved. In particular, the use of NGS highlighted the involvement of *Lactobacillus* in the digestion of barley, a finding that was not previously known from culturing and low throughput sequencing techniques. There were however some limitations of the study design. First, increasing the number of replicates used in NGS analysis may have improved the power to observe differences between barley varieties. With the cost of current NGS technologies decreasing, it is now feasible to analyze more sample replicates in study designs. Secondly, the low number of OTU reads used in microbiota analysis, particularly for the high grain diet, may have led to some rare OTUs being unobserved. As with sample costs, the costs of sequence depth per sample have also decreased since this study took place, and it is now attainable to achieve >20,000 reads per sample in NGS experiments. Lastly, bacterial contamination may have occurred throughout sample processing. To circumvent this in the future, blank samples (i.e. DNA extractions using only reagents, and no sample material) should be processed in parallel with substrate samples throughout DNA extraction and also sequenced. This will allow for evaluation of contaminating DNA from the lab environment and DNA extraction reagents.

5.0 Future Directions

Individual microbial identification and the function and interaction of microbial communities are important areas of study in nutrition because of the rumen microbiota's contribution to feed efficiency, ruminant productivity, and metabolic disorders. Traditionally, the study of rumen microbiology relied on culture-dependant techniques to identify and enumerate populations of microbes and led to approximately 300 to 400 species of bacteria, protozoa, and fungi being described within the rumen (Krause et al., 2007). Studies in molecular ecology suggested that this likely accounts for 10 to 20 % of the total microbial population in the rumen (Edwards et al., 2008). As a result, more recent technologies such as NGS of the 16S rRNA gene have suggested that there at least 300-400 different bacterial species residing within the rumen (Edwards et al., 2004; Yu et al., 2006). However, although information on bacterial diversity and relative abundance within the rumen provide insight into the microbial ecology, they are limited in defining the functional capacity of the community. Studies using 16S rRNA-based data tend to emphasize the most relatively abundant taxa and these data do not necessarily provide information on the metabolic function of the communities. Both growth rate and substrate utilization may not necessarily correlate with bacterial abundance in microbial communities (Kurm et al., 2017). In addition, Jousset et al. (2017) showed that rare species that may frequently go undetected even by molecular techniques may have important roles in community metabolism and function.

Shotgun metagenomic studies, which involve sequencing all DNA present in a sample, provides important information on the genetic potential of a microbial community, in addition to taxa present. Thus shotgun metagenomics studies would be useful in ruminant nutrition by

providing genomic information that could identify possible linkages between function and phylogeny, especially as it may relate to those microorganisms that cannot be cultured in the laboratory. Metagenomics may also allow for the identification of novel enzymes for other applications such as the development of feed additives. For example, metagenomic studies have been performed to characterize fibre-degrading enzymes in the rumen microbiota (Jose et al., 2017). Further, isolation and characterization of these enzymes could lead to feed additives with the potential to enhance plant cell wall digestion. However, while metagenomics studies do provide information on genes present in the rumen bacterial community, they do not necessarily convey the importance of these genes for substrate digestion. Complementing metagenomics with metatranscriptomics, which involves isolation and sequencing of RNA, or metaproteomics, which involves sequencing of proteins, allows for evaluating what genes are being expressed and potentially being translated into functional proteins. These data could be utilized to further evaluate biochemical pathways involved in the digestion of starch and fibre. For example, identifying rate limiting steps involved in the degradation of the protein matrix associated with corn could be useful in enhancing the ruminal digestion of starch in corn.

As sequencing technologies evolve, the ability to generate large amounts of data is becoming less expensive. Analyzing these data will become increasingly important to interpret what factors are important in rumen microbiology. Thus bioinformatics and the tools to evaluate data will be as important as sequence generation. Tools such as cloud computing and central data analysis centres would limit the constraints currently experienced with transferring and analyzing large data sets. In addition, a combination of microbiota analysis, metagenomics, metatranscriptomics, and metaproteomics, would provide a better understanding of rumen microbiology and nutrition by providing information on microbial abundances, genetic potential

of communities, and identification of novel proteins involved in digestion. These technologies will be important for future strategies to improve feed digestion, the productivity and health of ruminants.

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Supplementary Figure S1. Rarefaction curves comparing the number of reads with the number of phylotypes when heifers were fed A) low grain diet and B) high grain diet.